The Role of Costal2 and its Collaborators in Regulation of Ci Processing and in Mediation of Response to Hedgehog in Drosophila

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ABSTRACT

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Hedgehog (Hh) family proteins specify many cell fates in flies and mammals that depend on Hh concentration. Hh achieves differential expression of target genes by regulating the activity and stability of Cubitus interruptus (Ci) / Glioblastoma (Ci / Gli) transcription factors. Both modes of regulation require kinesin-like protein Costal2 (Cos2) (Kif7 in mammals) that acts in a complex with Fused (Fu) kinase. We used a group of *fused* (*fu*) alleles with truncations in the Fu-regulatory domain to confirm the importance of physical association between Cos2 and Fu for processing of Ci-155 to Ci-75 repressor in the absence of Hh. By specifically disrupting Cos2 interaction with Fu using Cos2ΔFu transgenes, we confirmed the importance of this interaction for stability of the Fu protein and for Ci processing when the transgene was expressed at natural levels (under control of genomic regulatory sequences). It is possible that Cos2/Fu interaction helps recruit processing-promoting kinases to Ci. We found no evidence for the importance of Suppressor of fused (Su(fu)), a protein known to limit Ci activity, in regulating Ci processing. Using a group of Cos2 variants under the control of genomic sequences, we found that expression of the wild type genomic Cos2 transgene (gCos2) or gCos2 deficient in Fu-dependent phosphorylation of Ser572 and Ser931 rescued animals to adulthood. Contrary to past observations of over-expressed phosphorylation variants, these gCos2 transgenes supported normal responses to Hh at the anterior-poster (AP) border of wing discs, a region of active Hh signaling. As shown

previously, Ser572 had a role in stabilization of Ci-155 by activated Fu kinase. gCos2ΔFu greatly reduced the response to Hh and was also unable to rescue animal viability. The transgene allowed for induction of *ptc-lacZ* in response to activated Fu (GAP-Fu) but did not respond to activated Smo (SmoD123), suggesting that it fails to mediate Fu activation in response to activated Smo . Normal accumulation of Smo in the regions of active Hh signaling in cos2-null wing discs expressing Cos2ΔFu also suggested that Cos2ΔFu does not affect the accumulation component of Smo activation. We propose that Cos2ΔFu is defective in promoting Fu activation through cross-phosphorylation of Fu molecules that requires physical interaction with Cos2. Similarly, a gCos2-S182N variant with impaired binding to Ci failed to rescue animal viability and was defective in Ci processing, but gCos2-S182N supported almost normal responses to Hh at the AP border. Ectopic induction of *ptc-lacZ* in anterior *cos2* clones with gCos2-S182N indicated a defect in limiting Ci activity possibly through physical interaction.

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Lastly, this work is done with support from my family members.

Chapter I

Introduction

Part I. Background and Motivation for Studying Hedgehog Signaling in Drosophila

The Family of Hedgehog Secreted Proteins is Essential for Developmental Patterning and Maintenance of Animal Tissues

During the development of multi-cellular organisms, Hedgehog (Hh) family proteins are responsible for specifying many cell fates. In each case a Hh protein is typically expressed and secreted from a localized cellular source (Jiang and Hui, 2008; Ogden et al., 2008). As the Hh signal travels several cell diameters away from its cellular origin, a Hh concentration gradient is established. The cells that lie within the range of Hh stimulation can often produce diverse developmental fates that depend on the concentration of the Hh signal in their local environment. Hh gradients are instrumental for patterning the nervous system as well as the appendages in mammals (Fig. 1.1). Thus, the gradient of mouse Sonic Hedgehog (Shh), a member of the mammalian Hh family, instructs the neural progenitor cells in the neural tube to assume the fates of distinct neuronal subtypes, and Shh also patterns the limb buds along the anterior-posterior axis (Jiang and Hui, 2008; Hui and Angers, 2011; Briscoe and Thérond, 2013). Consequently, defects in Hh signaling during mammalian embryogenesis can result in cranial malformations and faulty limb development (Ming et al., 1998). Human syndromes include common holoprosencephaly and Pallister-Hall syndrome that results in polydactyly (Lettice et al., 2002; Hill et al., 2007). In the mature organism, Hh mediates the regeneration of epithelial and neural tissues by maintaining and expanding stem and

progenitor cell populations (Beachy et al., 2004; Jiang and Hui, 2008). Improper regulation of Hh signaling can lead to excessive proliferation and tumorigenesis, contributing to common malignancies such as medulloblastoma and basal cell carcinoma (Taipale and Beachy, 2001; Beachy et al., 2004). It is therefore important to understand how Hh signaling is regulated in aspiration to prevent birth defects, to stimulate tissue repair and to treat prevalent cancers.

Figure 1.1. Examples of Mouse-Derived Contexts for Studying Hh Signaling (taken from Goetz and Anderson, 2010). As a consequence of Shh secretion from the floor plate (red circle) at the ventral side of the neural tube (grey doughnut with rectangle showing the ventricular cavity), precursors of ventral neurons differentiate into distinct neuronal cell types depending on the concentration of Shh (in the order of decreasing concentration: orange, V3; yellow, motor neurons; green, V1 and blue, V0). Secretion of Shh from the zone of polarizing activity (ZPA, not shown) in the limb bud specifies the digits in the adult limb. Response to Shh can also be assayed in tissue culture by using immune fluorescence and biochemical analyses (Goetz and Anderson, 2010).

The normal actions of Hh signaling in mammals have been studied most extensively in the developing neural tube and the developing limb bud, as summarized briefly below. The neural tube is an embryonic precursor to the central nervous system in mice and other mammals. First, Shh expressed in the notochord induces strong Shh expression in the adjacent most ventral region of the neural tube, known as the floor plate

(Yu et al., 2013). Then, Shh spreads from the floor plate cellular source to establish a gradient that specifies the fates of a variety of ventral neurons (Fig. 1.1). Ectopic activation of the Shh pathway causes expansion of ventral cell types in the neural tube, while reduction of Shh signaling causes dorsalization of the neural tube (Goodrich et al., 1997; Tuson et al., 2011).

In the developing limb Shh is critical for specifying digits (Chiang et al., 2001). Shh diffuses from the Zone of Polarizing Activity (ZPA) in the posterior limb bud towards the anterior. The Shh gradient specifies the number and identity of digits in the limb in a manner that depends on both the concentration of Shh and the time of exposure (Chiang et al., 2001; Harfe et al., 2004). Activation of the pathway causes the formation of extra digits (polydactyly), while reduction of Hh signaling causes a loss of digits.

Components of the Hh signaling pathway in vertebrates are localized to the primary cilium, a microtubule-rich structure characteristic of vertebrate cells. Close association between defects in cilia maintenance and defects in Hh signaling explains many defects observed in human ciliopathies (disorders due to structural or functional abnormalities of cilia) (Goetz and Anderson, 2010).

The Drosophila Wing Disc is an Excellent Model System for Studying Hh Signaling In Vivo

Drosophila is an organism where the core components of Hh signaling were first identified and where Hh signaling was initially most thoroughly studied (Fig. 1.2). In the next part of the introduction, I will explain the historically important role of Hh signaling in embryonic and larval segmentation. Afterwards, I will describe the role of Hh in

patterning the anterior wing imaginal disc, which is the focus of our studies. Lastly, I will mention relevant Drosophila cell lines and their applications.

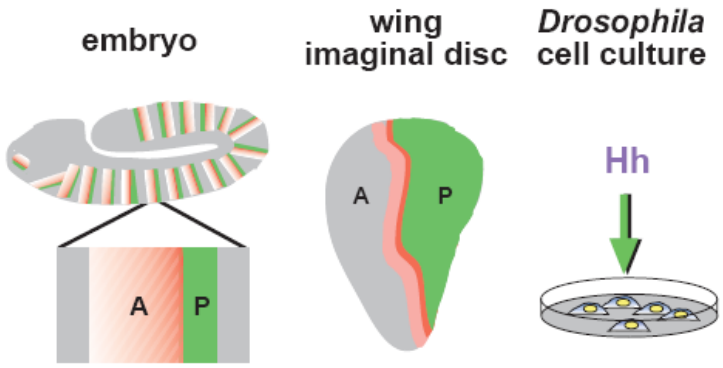


Figure 1.2. Hh Signaling Can Be Studied in a Variety of Drosophila-Derived Contexts (taken from Lum and Beachy, 2004). The Hh-secreting population of cells is denoted in green (P, posterior). The responding population is marked with red (A, anterior), where the color intensity reflects the local concentration of the Hh protein (Lum and Beachy, 2004).

Developmental Context and Techniques for Studying Hh Signaling in Drosophila

Hh was first identified as a gene that affects segment polarity of Drosophila larvae (Nusslein-Volhard and Wieschaus, 1980). The major role of Hh in Drosophila segmentation is to maintain the transcription of *wingless* (*wg*), a Wnt family signaling protein, at the compartment boundaries that separate the individual segments in the Drosophila embryo (Ingham, 1993; Ingham, 1998) (Fig. 1.3).

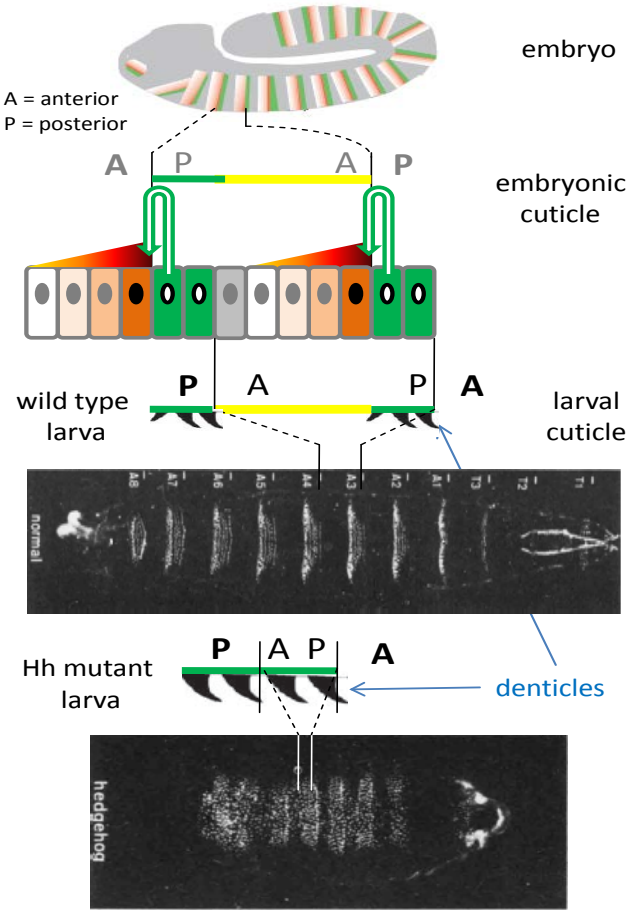


Figure 1.3. Hh Determines the Polarity of Larval Cuticular Segments (taken from Nusslein-Volhard and Wieschaus, 1980). The subdivision into anterior and posterior compartments takes place in the Drosophila embryo, which initiates the expression of the *en* selector gene in repeating stripes of posterior cells (P-cells, green) (Nusslein-Volhard and Wieschaus, 1980). P-cells produce and secrete Hh that travels across the embryonic compartment boundaries (green arrows) and maintains the transcription of *wg* in the adjacent stripes of anterior cells (orange) (Hidalgo and Ingham, 1990; Ingham and Hidalgo, 1993). Conversely, Wg signals to P-cells to maintain En, and consequently, Hh expression (van den Heuvel et al., 1993; Lawrence et al., 1996). Wg also moves anteriorly across each embryonic segment, specifying the fate of the ectodermal cells that secrete the larval cuticle (Dougan and DiNardo, 1992; Lawrence et al., 1996; Ingham, 1998). The more anterior cells form the naked part of the larval segments, while the posterior cells develop spiky cuticular projections or the denticles. Because *hh* -mutant embryos cannot maintain the expression of *wg* in

the embryonic segments, the naked cuticle part of each larval segment is lost, while the remaining denticled parts produce the phenotype that accounts for the name of the *hh* gene (Ingham and McMahon, 2001).

The division into anterior and posterior compartments persists in the imaginal discs, or the sacs of cells that arise in the embryo and grow beneath the larval cuticle to originate the appendages and other superficial structures of the adult fly during metamorphosis (Fig. 1.4A). Namely, *en* and *hh* continue to be expressed in the posterior cells, while the secreted Hh moves into the anterior compartment and induces distinct transcriptional responses in the adjacent stripe of the anterior cells (i.e. the AP border) in a dose-dependent manner (Fig. 1.4B).

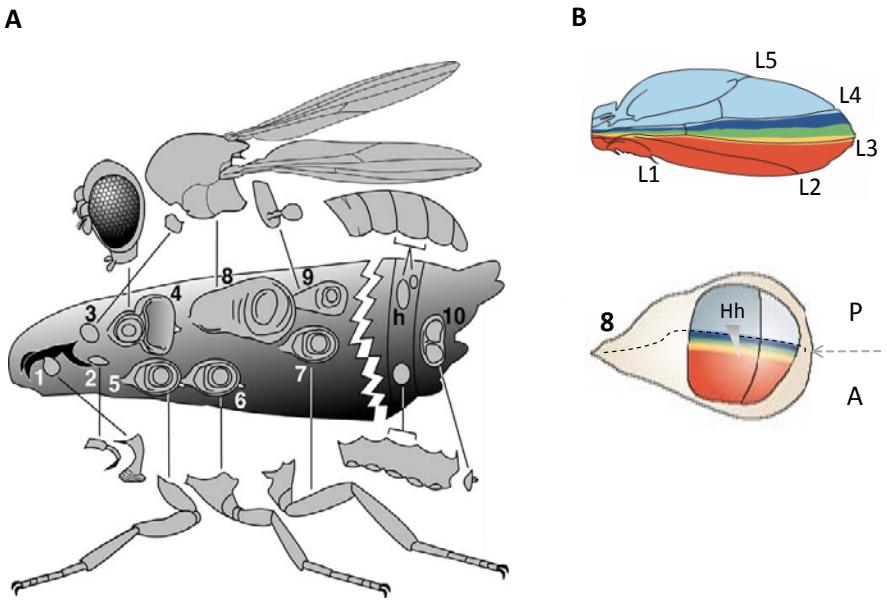


Figure 1.4. Developmental Context of Hh Signaling in *Drosophila* Wing Disc (taken from Held, 2002 & Hooper and Scott, 2005). (A) Imaginal discs and the structures that they produce in the adult fly (Held, 2002). Imaginal discs are the groups of cells that derive from the embryo and consist of two distinct populations, or compartments of about the same size. During larval development, the discs grow approximately 1000-fold from a starting population of roughly 50 cells (Johnston, 1998). Disc number 8

gives rise to the adult wing. Here, the discs are shown in Dorsal-Ventral (DV) orientation with Dorsal side pointing to the left, whereas the discs are typically presented with Dorsal side pointing to the top of the page and anterior to the left. (B) Hh pathway response in Drosophila wing disc (Hooper and Scott, 2005). Hh traverses the AP boundary (dotted line) and induces the concentration-dependent expression of its target genes at the AP border (A, red; P, light blue). Blue stripe indicates the region of highest Hh concentration, which results in transcription of anterior *engrailed* (*en*) and patched (*ptc*). Green stripe shows the region of moderate Hh concentration, which expresses lower level of *ptc* and *decapentaplegic* (*dpp*). Yellow stripe represents the area of low hedgehog concentration, marked by lower levels of *dpp* and expression of *iroquois* (*iro*). Hh transcription targets pattern the region between veins L3 and L4 in the adult wing (top), and Dpp also forms a long-range gradient in the wing disc to pattern the rest of the AP axis (Nellen et al., 1996).

Several Genetic Approaches are Available to Manipulate Hh Pathway Components In Vivo

Studying Hh signaling in the Drosophila wing disc benefits especially from the available genetic approaches such as mosaic analysis (Theodosiou and Xu, 1998; Lee and Luo, 2001) and targeted gene expression of protein variants (Duffy, 2002). In clonal analysis, site-specific recombination events give rise to clones of cells that are homozygous for a mutant genotype in a heterozygous background (Fig. 1.5A). These clones can be marked negatively, by the loss of a certain marker such as GFP, or positively, by the expression of a certain marker. The targeted gene expression strategy achieves tissue-specific expression of a target gene in Drosophila by combining yeast transcriptional activator GAL4 under control of tissue-specific genomic enhancer with a target gene preceded by UAS (Upstream Activating Sequence) GAL4 binding sites (Brand and Perrimon, 1993). The strategy is applicable when a gene product is expressed throughout the wing disc using C765-GAL4 driver (Flybase) or in pre-defined regions of the wing disc (Brand and Perrimon, 1993). Alternatively, expression of the gene can be

limited to clones of cells undergoing a specific recombination event, using the MARCM (mosaic analysis with a repressible cell marker) technique (Lee and Luo, 2001) (Fig. 1.5B).

The original application of the MARCM technique was to label neurons in the Drosophila CNS (Lee and Luo, 2001). The technique was developed to suit the anatomy of neuronal tissue with densely packed neuronal processes that could only be successfully resolved if positively labeled in small populations. The Drosophila wing disc has a straight-forward cellular architecture that consists of a basal layer of columnar epithelium continuous with the apical surface of the peripodial membrane or squamous epithelium (Aldaz et al., 2013). However, the MARCM technique is very useful in the wing disc because, besides specifically labeling the clones, it restricts the expression of transgenes to the clones and avoids potentially confounding effects of ubiquitous transgene expression on the growth and maintenance of disc tissue. The technique was recently expanded to accommodate a stochastic multicolor labeling approach, with FRT-flanked cDNAs for UAS transgenes of different fluorescent proteins arranged in tandem (Hadjieconomou et al., 2011). The resulting genetic system, Flybow, has a greater utility for tracing cell lineage and visualizing neuronal junctions than for labeling wing disc clones, where sparse labeling has no clear advantage.

Finally, embryo-derived S2 and KC cell lines are available for co-localization assays, gene silencing experiments and FRET (Fluorescence Resonance Energy Transfer) analysis. These lines do not express Ci but are easy to grow (Schneider, 1972) and are commonly used to study the interactions between Hh signaling proteins (Lum et al., 2003b; Zhang et al., 2005; Zhou and Kalderon, 2010). For example, S2 cells were used for immunoprecipitation (IP) experiments that showed a Hh-dependent decrease in the ability

of Cos2 to scaffold Ci processing-promoting kinases (Zhang et al., 2005). These cells were also used for co-localization and FRET analysis to show increased cell surface localization and a change in conformation of Smoothened (Smo) signal transducer in response to Hh (Jia et al., 2003; Liu et al., 2007; Fan et al., 2012). Wing-disc derived Cl8 cells, also frequently encountered in the literature (Stegman et al., 2000; Lum et al., 2003b; Ruel et al., 2007), are used to study Ci activity and its response to Hh (Fig. 1.2). These cells express Ci endogenously but are harder to maintain because they require a more complex medium supplemented with fly extract and are very sensitive to the density of the cells (Peel et al., 1990). The Therond group used Hh-expressing Cl8 cells in assays of Ci activity with a *ptc*-luciferase reporter to demonstrate the importance of Fu-dependent Cos2 phosphorylation for Ci activation (Ruel et al., 2003; Ruel et al., 2007; Ranieri et al., 2012). They were also the only group to detect Hh-dependent dissociation of Ci from Cos2 in IP assays in Cl8 cells (Ruel et al., 2003).

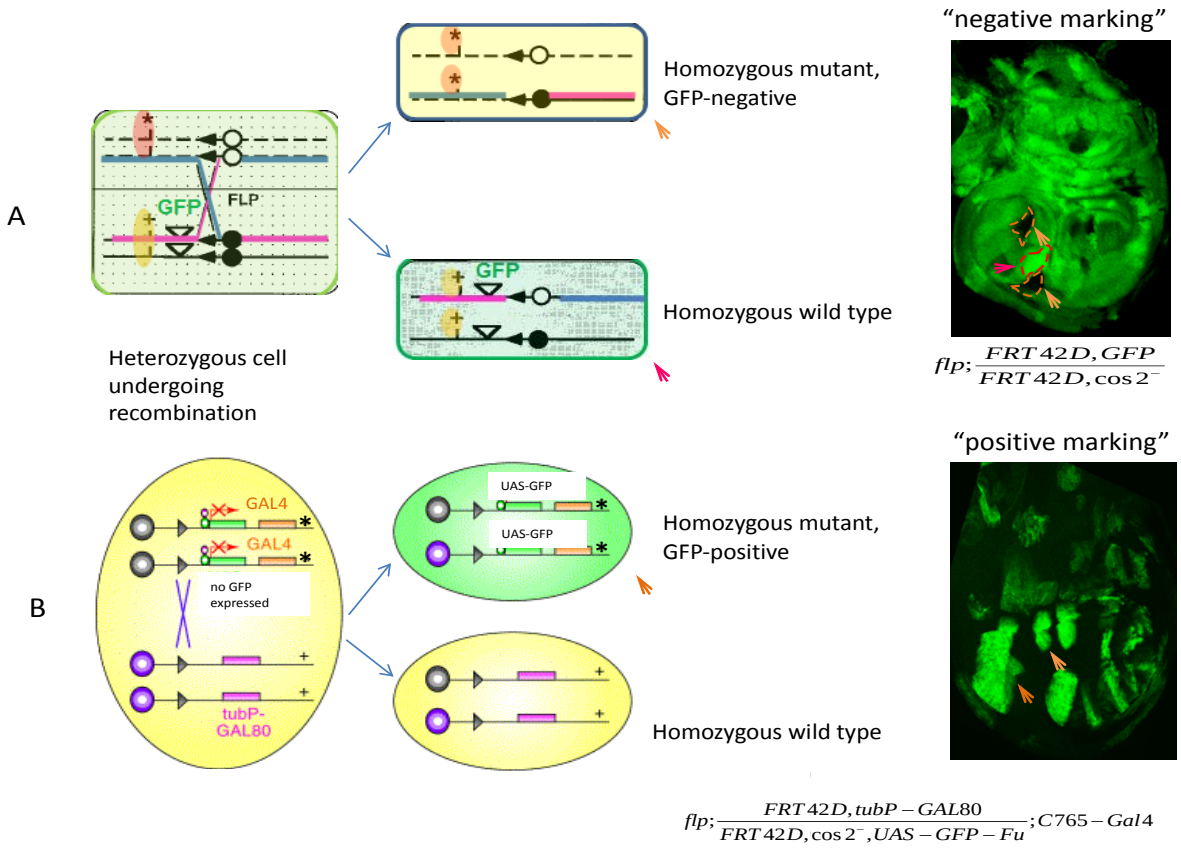


Figure 1.5. Genetic Approaches Available in Drosophila (taken from Theodosiou and Xu, 1998 & Lee and Luo, 2001). (A) FLP / FRT system. The yeast FLP / FRT system for site specific recombination is used routinely in Drosophila to induce mitotic recombination events in mosaic or clonal analysis (Theodosiou and Xu, 1998). The expression of FLP recombinase under a heat shock-inducible promoter is stimulated typically by heat-shocking the larvae for 1-hour at 37° C. FLP catalyses the recombination between the flippase recognition target (FRT) sites that are integrated on homologous chromosome arms with either the mutation of interest or a cell marker. During mitotic recombination, a mother cell that is heterozygous for the mutation segregates a daughter cell that carries two copies of the mutation as well as the wild-type cell (i.e. the twin spot). In negative marking, the mutant clone that derives from the homozygous mutant daughter cell is identified by the absence of a marker, such as GFP, while the twin spot carries two copies of that marker. The negative marking system is used to construct *cos2* mutant clones in this example (orange arrows and outlines). Red arrow and outline shows one of the GFP-positive sister clones that are wild type for Cos2. (B) FLP / FRT system: MARCM. A mutant clone that is produced by mitotic recombination can be identified by

the presence of a certain marker such as GFP using the MARCM method (Lee and Luo, 2001). In this method, GAL4-driven expression of a marker and any UAS-transgene present is prevented by ubiquitous expression of GAL80, an inhibitor of GAL4-mediated transcription. We also used the C765-GAL4 enhancer trap (Flybase.org) to induce GAL4 expression throughout the wing disc. When a mutant clone loses GAL80 expression as a consequence of mitotic recombination, the clone can be identified by the expression of a marker and can also express the UAS-transgene. The restriction of transgene expression to a positively-marked mutant clone is important, because the ubiquitous expression of certain transgenes is problematic for the growth of the disc. Here, a positive marking experiment restricts the expression of UAS-GFP-Fu protein to *cos2* mutant clones (orange arrows).

Part II. Overview of the Hh Signaling Pathway

Basic Principles of Hh Signaling are Conserved

Hh stimulates Ci/Gli family zinc (Zn) finger transcription factors (Fig. 1.6) by modifying their concentrations and activities to favor transcriptional activation over repression of target genes that contain Ci/Gli binding sites (Huangfu and Anderson, 2006). Mammals express three homologs of Ci: Gli1, Gli2 and Gli3, of which only Gli2 and Gli3 can be processed, while Gli1 acts predominantly as an activator. The stabilization and the accumulation of Ci is crucial for the expression of Hh targets, because they are actively repressed by its processed form (CiR) in the absence of Hh (Methot and Basler, 1999) (Fig. 1.7A).

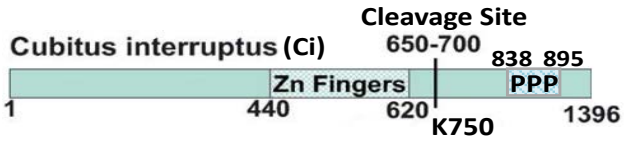


Figure 1.6. Important structural elements of Cubitus interruptus (Ci) (adapted from Ogden et al., 2004). Important structural domains and phosphorylation sites are indicated with numbers representing amino acids. Zn finger DNA-binding domain ("Zn Fingers"); phosphorylation cluster ("PPP"); K750 substrate of ubiquitination and the Cleavage Site of arrested proteolysis (see text for detail).

Mutational analysis of a Ci phosphorylation cluster revealed that hierarchical phosphorylation by protein kinase A (PKA), glycogen synthase kinase 3 (GSK3) and Casein Kinase 1 (CK1) creates a binding site for Slimb (Smelkinson and Kalderon, 2006). Slimb is a homolog of vertebrate βTRCP that functions in the context of Skip 1/ Cullin 1/ F-box (SCF) complex to recruit E3 ubiquitin ligase (Jiang and Struhl, 1998). Therefore, Ci phosphorylation promotes the association between Ci and Slimb, which leads to Ci ubiquitination and recruitment to the proteasome (Dai et al., 2003; Jia et al., 2005; Smelkinson et al., 2007). However, Ci (Ci-155) is not proteolyzed completely but undergoes limited proteolysis (processing) of the C-terminal part, which yields a 75-kD truncated form of Ci (Ci-75). Ci processing was observed in a western blot analysis of Cl8 cell extracts, where the generation of Ci-75 was blocked by proteasome inhibitors (Chen et al., 1999). IP of Ci variants from KC cells showed that the Zn finger region and Lysine750 are necessary for generation of Ci-75. It was proposed that Lysine750 (K750) is a substrate for Ci ubiquitination (Wang and Price, 2008) (Fig. 1.6). The N-terminal portion of Ci that precedes the "cleavage site", likely a site of arrested proteolysis from the C-terminus by the proteasome is shared by the full-length form of Ci and the Ci-75processed form (Fig.

1.7A). Hh prevents Ci processing at the AP border, which is evident from the elevation of full-length Ci-155 stained by the 2A1 antibody that recognizes an epitope C-terminal to sequences retained in Ci-75 (Fig. 1.7). Ci-75 acts as a repressor (CiR) of Hh transcription targets that include *hh*, and ectopic expression of *hh* was observed in anterior *ci* clones (Domínguez et al., 1996; Methot and Basler, 1999).

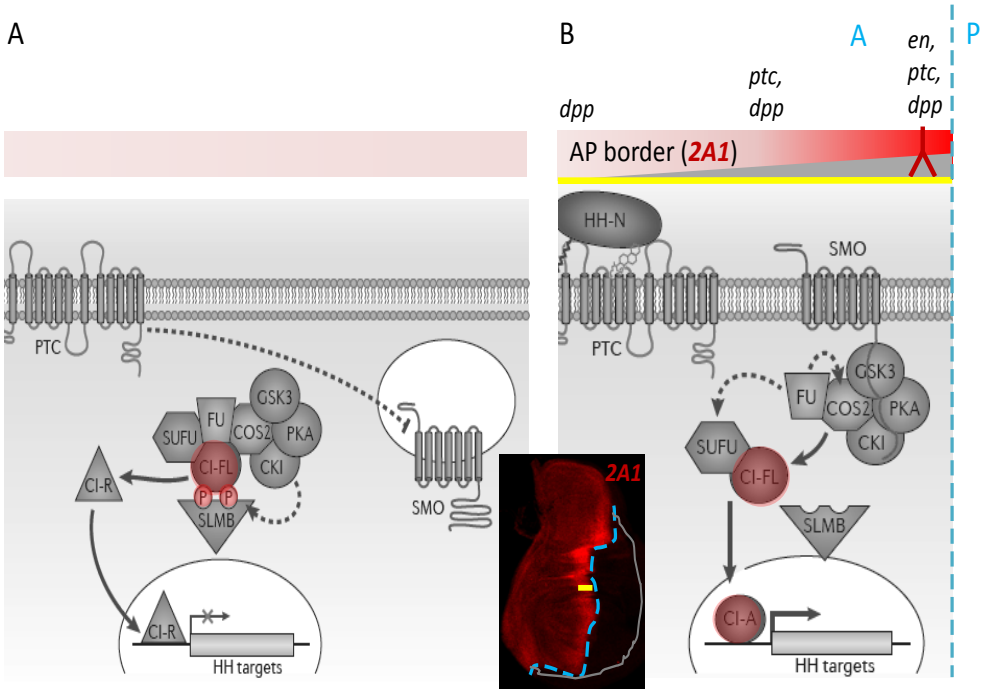


Figure 1.7. Molecular Context of Hedgehog Signaling in Drosophila Wing Disc (pathway diagram taken from Ingham et al., 2011)**.** A diagram of Hh signaling in Drosophila Wing Disc (inset). Hh (more specifically Hh amino-terminal polypeptide [HH-N]) crosses the AP boundary (blue dotted line) to regulate the expression of transcription target genes at the AP border (yellow) by controlling the abundance and the activity of its transcription factor Ci. Without Hh, full-length Ci is processed to a 75-kD repressor form (Ci-R or Ci-75). The level of Ci can be assayed by immunostaining with 2A1 monoclonal antibody (red). 2A1 antibody is a convenient tool for following Ci proteolysis, because its epitope is located within the C-terminal region of Ci that contains transcription activation domain and is lost during the processing step (Ogden et al., 2004). Far anterior cells show decreased 2A1 staining because of Ci processing to Ci-R. (A) *Without Hh*, Patched (Ptc) inhibits Smoothened (Smo). A Hh signaling complex (HSC) that includes Cos2, Fu, and

possibly Sufu regulates the phosphorylation of Ci by protein kinase A (PKA), casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3) (Hooper and Scott, 2005). Ci phosphorylation by PKA, CK1 and GSK3 creates a binding site for Slimb (Slmb), a component of Skp1 / Cullin1 / F-box (SCF) complex that recruits E3 ubiquitin ligase and targets Ci for partial proteolysis (Smelkinson and Kalderon, 2006). Full-length Ci (Ci-FL) is partially processed to a repressor form (Ci-R) that enters the nucleus and silences the transcription of Hh target genes. (B) Hh protein inhibits Ptc, allowing the activation of Smoothened (Smo). Smo becomes phosphorylated by PKA and CK1, and translocates to the membrane from the vesicular pool. Phosphorylation of Smo changes the conformation of Smo C-terminus, allowing for a closer interaction between Smo and Cos2 (Fan et al., 2012). Complexes that contain Smo together with the phosphorylated forms of Cos2 and Fu accumulate at the membrane (Kalderon 2004). Activated Fu phosphorylates Cos2 and facilitates the detachment of Ci from the HSC and the processing-promoting kinases (Ruel et al., 2007; Ranieri et al., 2012). Ci proteolysis, and hence the generation of the Ci repressor form, arrests; Ci-FL accumulates. Certain Hh targets such as *dpp* and ptc become active (Methot and Basler, 1999). However, Sufu limits the activity of Ci, possibly in part by opposing the nuclear entry of Ci (Methot and Basler, 2000). *At high concentration of Hh*, Fu kinase inhibits Sufu, allowing Ci to translocate into the nucleus and act as a transcription activator (Ohlmeyer and Kalderon, 1998). The activator form of Ci (Ci-A) induces the transcription of such targets as anterior *en* (Ingham et al., 2011).

Without Hh, Ci can be found in a cytoplasmic Hh signaling complex (HSC), which includes the kinesin-like protein Costal2 (Cos2), the serine / threonine kinase Fused (Fu) and can also include Suppressor of Fused (Sufu), a protein of unknown function (Robbins et al., 1997; Stegman et al., 2000). A similar complex was not yet identified in mammals, but Sufu, microtubule (MT) – associated intraflagellar transport proteins (IFTs) and Kif7, a Cos2 ortholog, are important for Gli regulation, because the removal of either Sufu, IFTs or KIF7 activates the pathway to some degree in mice (Huangfu and Anderson, 2005;

Cheung et al., 2009). The HSC complex mediates proteolytic processing of Ci into CiR (Fig. 1.7 A).

Cos2 is essential for processing in Drosophila, and de-repression of *ptc* in anterior *cos2* clones is consistent with the negative role of Cos2 in regulating the Hh pathway (Sisson et al., 1997; Wang and Holmgren, 1999). Similarly, reducing the level of *cos2* mRNA in zebrafish embryos results in expansion of *ptc1* domain in muscle precursor cells that are patterned by Shh (Tay et al., 2005). Loss of Kif7 in mice produces limb phenotypes that are consistent with pathway activation, and western blots of extracts from *kif7* limbs show a decrease in production of Gli3 repressor relative to full-length Gli3 (Litingtung et al., 2002; Ingham and McMahon, 2009).

Ci proteolysis is blocked by the activation of Smoothened (Smo), a seven-pass transmembrane protein of the G-protein coupled receptor (GPCR) superfamily. Smo activation entails phosphorylation, accumulation at the plasma membrane and a change in conformation (Denef et al., 2000; Wang et al., 2000; Zhao et al., 2007; Shi et al., 2011). Activating phosphorylation of Smo is accomplished by PKA and CK1 in flies and also by G-protein receptor kinase 2 (Gprk2) in both flies and mammals (Aikin et al., 2008). In the absence of Hh, Ptc, a 12-span Niemann-Pick C (NPC) family transporter, keeps Smo in the inactive state through an unknown mechanism which may involve transport of a small molecule inhibitor of Smo (Taipale et al., 2002; Rohatgi and Scott, 2007) (Fig. 1.7 B).

When Hh inhibits Ptc, Smo-Cos2 complexes accumulate at the plasma membrane, and Ci is no longer processed (Lum et al., 2003b). However, the association of Ci with Sufu, and likely Cos2 also, limits the activity of Ci; only blocking Ci proteolysis fails to

activate some Hh target genes (Methot and Basler, 2000; Smelkinson et al., 2007). Similarly, Sufu limits the activity of Gli proteins in mammals. Besides facilitating Ci-155 processing in anterior cells, Cos2 is also required for normal responses to Hh at the AP border: Cos2 stimulates Ci activation, and *cos2* mutant clones at the AP border (in the presence of Hh) lose the expression of anterior *en* (Wang, G. et al., 2000) (Fig. 1.7B). Like Cos2, vertebrate KIF7 proteins are important for high level Hh signaling and also prevent Hh target gene activation in the absence of ligand (Liem et al., 2009). The pathway details discussed so far are generally applicable to flies and mammals.

Fu

Fu protein plays a dual role of promoting Ci activation (Ohlmeyer and Kalderon, 1998; Methot and Basler, 2000) and regulating Ci proteolysis (Methot and Basler, 2000; Lefers et al., 2001) in Drosophila (Fig. 1.7; Table 1.1). The Fu Serine/Threonine (S/T) kinase consists of a kinase domain followed by a regulatory domain of similar length. Kinase activity is essential for Fu activation but only the regulatory domain is required for Ci processing in anterior wing disc cells. The contribution of Fu to Ci processing is evident from an elevation of full-length Ci in *fu* mutant clones (Lefers et al., 2001).

In response to Hh and Smo activation, Fu kinase is activated. Fu kinase inhibits Sufu, which allows Ci to activate the remaining transcription targets, including *en* (Ohlmeyer and Kalderon, 1998). However, phenotypic studies in mice showed that the mammalian Fu ortholog (mFu) is dispensable for Hh signaling. Another peculiar characteristic of the mammalian Hh pathway is that all of the known pathway components localize to non-motile cilia, microtubule (MT)-rich organelles found in most interphase

mammalian cells (Huangfu and Anderson, 2006). In fact, intraflagellar transport proteins (IFTs) that regulate Gli activity in mice also produce and maintain non-motile cilia (Huangfu and Anderson, 2005). Studies in planaria, a common evolutionary ancestor of vertebrates and Drosophila, attribute ciliary localization of Hh signaling in mammals to the ancestral function of Kif7 homologs in ciliary maintenance (Rink et al., 2009).

Cos2

Kinesin-like protein Costal2 (Cos2) promotes both Ci processing (in the absence of Hh) and Ci activation (in the presence of Hh) in Drosophila. The basic evidence for Cos2 regulation of Ci proteolysis from 2A1 immunostaining is that Ci levels are increased in anterior *cos2* mutant wing disc clones (Sisson et al., 1997). Cos2 is not a typical kinesin, because its motor domain is only about 30% identical to that of a typical kinesin (e.g. Kif3). However, live imaging in S2 cells that expressed GFP-Cos2 has revealed that Cos2 did exhibit movements similar to other motor proteins tracking along microtubules (Farzan et al., 2008). Like several other kinesins (e.g. BimC, KlpA), Cos2 contains two kinesin heavy chains (KHCs) that include a globular motor domain for ATP proteolysis, heptad repeats for dimerization and a cargo domain for vesicle binding (Fig. 1.8). Cos2 regulates the fate of Ci by connecting Ci with appropriate Hh signaling components depending on Hh concentration.

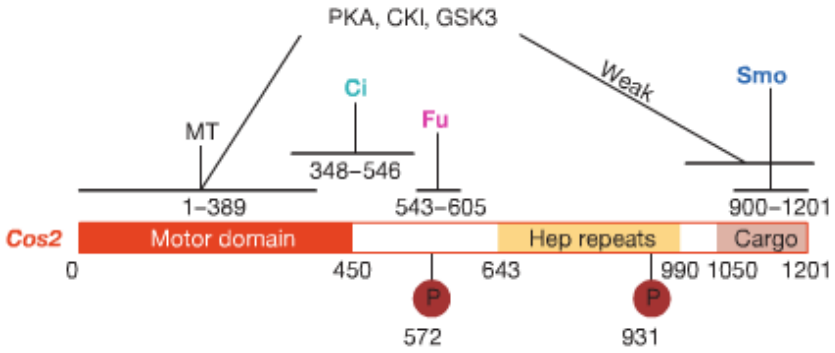


Figure 1.8. Topology of Costal 2 (Cos2) (taken from Aikin et al., 2008).

Important binding domains, structural domains, and phosphorylation sites are indicated. Microtubule-binding domain ("MT"); ATP-driven Motor domain; Heptad repeats for dimerization ("Hep repeats"); Cargo domain for vesicle binding; binding sites for Ci (Monnier et al., 2002; Wang and Jiang, 2004), Fu (Ruel et al., 2007), Smo (Ruel et al., 2003), kinases PKA, CK2 and GSK3 (Zhang et al., 2005); Fu-dependent phosphorylation sites Ser572 and Ser931 (Nybakken et al., 2002).

Without Hh, Cos2 scaffolds Ci with processing-promoting kinases PKA, CK1 and GSK3. Immunoprecipitation (IP) and yeast two-hybrid analyses showed that Cos2 also has direct interactions with Ci (Fig. 1.10) (Wang and Jiang, 2004; Zhou and Kalderon, 2011). Since Cos2 binds tightly to Fu, Cos2 may be indirectly recruited to Ci because Su(fu) binds to both Fu and Ci. Cos2 regulation of Ci processing as part of the cytoplasmic complex is the subject of Part III.

IP of Cos2 from S2 cell extracts demonstrated the ability of Cos2 to scaffold proteolysis-promoting kinases PKA, CK1 and GSK3, but the affinity of Cos2 for the kinases decreases in Hh-stimulated S2 cells (Zhang et al., 2005). There are conflicting reports on whether or not Hh also reduces the binding affinity of Cos2 for Ci (Ruel et al., 2003; Ruel et al., 2007; Lum et al., 2003b; Zhang et al., 2005).

Hh potentiates the association of Cos2 with the C-terminal tail of Smo (Jia et al., 2003). Activated Smo recruits Cos2-Fu complexes to the membrane, where Fu kinase becomes activated and phosphorylates Cos2 on Ser572 and Ser931 (Zhou and Kalderon, 2011; Ranieri et al., 2012). The positive role of Cos2 in Hh signaling is explained by its role in the activation of Smo and Fu (Zhou and Kalderon, 2011; Ranieri et al., 2012). Phosphorylation of Cos2 itself contributes to Ci activation on the basis of several tests in tissue culture cells and limited in vivo tests (Ruel et al., 2007; Ranieri et al., 2012). In addition, phosphorylated Cos2 has been reported to have lower affinity for Ci and for Smo (Lum et al., 2003b; Ruel et al., 2003; Ruel et al., 2007). Studying the role of Cos2 in Ci activation is the subject of Part IV.

Part III. The role of the Hh Signaling Complex (HSC) in Ci processing

HSC mediates Ci processing without Hh and dissociates in response to Smo activation (Fig. 1.9). Incomplete knowledge of HSC composition and its change in response to Hh contributes to two fundamental gaps in the understanding of Hh signaling pathway: 1) how is Ci proteolysis promoted by Cos2? and 2), how is Ci proteolysis inhibited by Smo? A more complete understanding of the HSC could potentially answer these questions. In this section, I will address the changes in composition in HSC in response to Hh, and then talk about the connections that are known to exist between its components.

I will first describe what is known about the composition of HSC and its change in response to Hh. Then I will talk about the effect of HSC protein interactions on Ci

processing in the following order: 1) Cos2-Ci interactions; 2) *fu* alleles and Sufu removal; 3) Cos2-Fu interactions (Fig. 1.9).

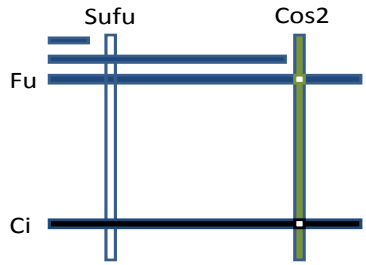


Figure 1.9. Cos2 Mediates Ci Processing as a Part of Cytoplasmic Transduction Complex

Hedgehog Signaling Complex (HSC) contains Fu and may also contain Sufu in addition to Cos2. Without Hh, the complex is known to exist in trimeric (Fu-Cos2-Ci) and tetrameric form (Fu-Cos2-Ci-Sufu), and also as Sufu-Ci that retains Ci in the cytoplasm but does not mediate processing. The diagram shows the connections that are known to exist between the individual components of the complex. Each intersection may represent multiple binding interactions, which is definitely true of Cos2 and Ci (Zhou and Kalderon, 2010). We attempted to disrupt the transduction complex by removing Sufu (white bar), using Cos2 without the binding site to Fu (white square on a green bar), using Ci without some of its binding sites for Cos2 (white square on a black bar), and by using various alleles of Fu that produce shorter proteins that lack a C-terminal Cos2-binding domain (blue lines).

Changes in HSC Composition with and without Hh

The Hh signaling complex (HSC) varies in protein composition independently of Hh stimulation and shows some consistent changes in response to Hh. Hh-dependent change in composition is most readily observed in cell culture, which is why a substantial amount of information about the HSC comes from in-vitro binding assays. The significant limitation of these assays is that they often involve protein over-expression and cannot reliably reproduce the stoichiometry of in-vivo complex formation. The physiological

relevance of findings from over-expression studies is established only if they are verified by studies that use endogenous proteins. Nevertheless, there are several core observations that are consistent and relevant to our work.

Hh-Regulated Association between Cos2 and Microtubules; Su(fu) Enrichment

A pioneering study of the HSC in S2 cells detected a trimeric complex that consisted of Cos2, Fu and Ci (Robbins et al., 1997). Hh stimulation induced Cos2 and Fu phosphorylation. These three proteins precipitated with microtubules (MTs) under MT-polymerizing conditions, but in lesser amounts when the cells were treated with Hh. After confirming the physiological relevance of the interaction between the complex and MTs in Drosophila embryos, Robbins postulated a Hh-dependent dissociation of the trimeric complex from MTs (Robbins et al., 1997). Melanie Stegman used Cl8 cells to demonstrate that Sufu can join the trimeric complex with or without Hh (Stegman et al., 2000). However, Sufu could not be detected in the embryo-derived MT fractions which contained Cos2, Fu and Ci. Both S2 and Cl8 cells express Hh signal transduction components such as Ptc, Smo and Cos2, but only Cl8 cells express Ci endogenously, which may have facilitated the discovery of the tetrameric complex.

Later, Lawrence Lum also used IP with an antibody to Sufu in Cl8 cells to detect a modified version of Stegman's tetrameric complex in Hh-stimulated cells, where Sufu was phosphorylated and Fu and Cos2 were present in lower amounts (Lum et al., 2003b). Without Hh, Sufu associated predominantly with Ci alone.

Dissociation of HSC in Response to Smo Activation

Lawrence Lum used an IP assay in Cl8 cells to show that Smo can also join the trimeric complex even in the absence of Hh (Lum et al., 2003b). These complexes accumulated further in response to Hh. Time-course western blot analysis of extracts from Hh-treated Cl8 cells showed accumulation of phosphorylated forms of Smo, Cos2 and Fu. The extracts were subjected to differential centrifugation to reveal Hh-dependent changes in localization of the Smo-Cos2 complex (Ruel et al., 2003). The complex was associated with vesicles without Hh and was enriched in the plasma membrane fraction in the Hh-treated condition.

IP of cell extracts showed that the complex also undergoes a Hh-dependent change in composition. First, IP of Cos2 from S2 cells showed that Hh decreases the affinity of Cos2 for proteolysis-promoting kinases (Zhang et al., 2005). Second, IP of Cl8 cell extracts with Smo and Cos2 antibodies showed that Ci is not present in the complex in Hh-treated cells (Ruel et al., 2003). Although Hh may change the amount of Ci in Smo-Cos2 complexes, the nature of this change is poorly understood. Indeed, a similar IP experiment by *Lum et al.* showed an opposite result where Ci accumulated in Smo-Cos2 complexes in response to Hh. Another IP experiment with Myc antibodies against Myc-Smo in extracts of S2 cells transfected with Ci failed to see Ci in a complex with Smo even without Hh, which was attributed to dynamic nature of Ci interaction (Jia et al., 2003). This set of findings suggests that Hh promotes stabilization of Smo-Cos2 interaction and may also affect Cos2 association with Ci (Kalderon, 2004).

The next set of studies address the molecular mechanism of Cos2-Ci dissociation. Laurent Ruel showed in Cl8 cells that Cos2 dissociation from Ci is impaired if Ser572 of Cos2 is mutated to Ala so as to prevent phosphorylation (Ruel et al., 2007) (Fig. 1.8). On the contrary, when Ser572 is mutated to Asp to mimic phosphorylation, Cos2-S572D precipitates with equally low amounts of Ci independent of Hh stimulation. The signal from antibody to phosphorylated Ser572 (pSer572) was absent in the extracts prepared from Hh-untreated cells and eliminated in Hh-treated cells that were transfected with kinase-dead form of Fu (Fu-KD) where a point mutation that changes Gly13 into Val prevents Fu kinase activation (Liu et al., 2007). *Ruel et al*. therefore concluded that Fu-dependent phosphorylation of Cos2 on Ser572 in response to Hh leads to Ci dissociation from Smo-Cos2 complex. Replacing the codon for Ser572 with Ala on a Cos2 transgene prevents Ci accumulation in *cos2* clones in response to activated Fu (Fu-EE), suggesting that phosphorylation of Cos2 on Ser572 also affects the stability of Ci (Zhou and Kalderon, 2011).

Recent findings linked Cos2 phosphorylation on Ser931 to high-level pathway activity and established the importance of Smo-Cos2 interactions for pathway activation (Ranieri et al., 2012). When antibody to Ser931 (pSer931) was compared to pSer572 with respect to signal specificity, both signals were dependent on Hh and the activity of Fu-kinase. However, pSer931 signal distribution at the AP border of imaginal discs was restricted to the stripe of high pathway activity (4-5 cells), while pSer572 signal encompassed the entire region of Hh signaling (AP border, 12-15 cells). IP of phosphorylated Cos2 with antibody to phosphorylated Ser931 (pSer931) from Cl8 cells showed increased binding of Cos2-S931P to Smo. In contrast, antibody to pSer572 showed

decreased binding of Cos2-S572P to Smo compared to wild type Cos2. Smo activation leads to dissociation of the HSC complex and potentially alters Cos2 properties to support Ci activation. The details of Cos2 activation will be discussed in Part IV.

Components of the HSC Complex and Their Interactions

IP and yeast two-hybrid analyses revealed two strong binding sites for Cos2 on Ci, N-terminal (CDN) and C-terminal (CORD) to the Zn finger domain (Wang, G. et al., 2000; Wang and Jiang, 2004) (Fig. 1.10). Binding assays of GST-Ci variants to HA-Cos2 showed that the last three Zn fingers of Ci can also bind Cos2 (Zhou and Kalderon, 2011). Cos2 is likely to mediate Ci proteolysis through physical interaction with Ci. In this section, I will describe the topology of Ci and the protein components of the HSC complex that determine the fate of Ci (Fu, Sufu and Cos2). Then I will talk about the effect of their interactions on Hh signaling.

Cos2 and Ci

The N-terminal portion of Ci that precedes the "cleavage site" (CS) contains a zinc finger (Znf) DNA-binding domain and nuclear localization signals (Fig. 1.10A). The C-terminal half of Ci that is absent from CiR contains nuclear export signals (Chen et al., 1999; Wang and Holmgren, 2000), a cluster of phosphorylation sites that stimulate the association with E3 ubiquitin ligase through Slimb adaptor protein (Smelkinson and Kalderon, 2006; Smelkinson et al., 2007) and a protein binding domain for the co-activator CBP (CREB (cAMP response element binding protein) -binding protein) (aa 1020 – 1160, not shown) (Akimaru et al., 1997; Ogden et al., 2004).

Zhou and Kalderon tested the physiological importance of Ci regions that were identified in yeast two-hybrid and GST binding assays to interact with Cos2 and Sufu (Methot and Basler, 2000; Wang and Jiang, 2004) by using Ci deletion variants. These Ci variants included a C-terminal Myc tag and were expressed evenly throughout the wing disc. Consequently, Myc staining should indicate the levels of unprocessed Ci and hence the efficiency of processing. For wild-type Ci-Myc the levels of Myc are higher at the AP border and in posterior cells than in anterior cells because Ci processing occurs only in anterior cells. However, the difference is quite small because high levels of Hh promote Ci-155 degradation (as opposed to processing) through the Cul3/Hib pathway in AP border and posterior cells (Zhang et al., 2006). From Myc staining simultaneous removal of the Zn finger (506-613) and CORD (941–1065), carboxyl C-terminal domain (Wang and Jiang, 2004), binding sites for Cos2 on Ci resulted in elevated anterior Ci levels, implying that Ci processing is inhibited, while each deletion alone did not inhibit Ci processing. CDN deletion also did not impair Ci processing alone or together with CORD deletion (Ci ΔCDNΔCORD). Surprisingly, deletion of the CORD domain alone or together with CDN enhanced Ci processing in anterior cells, leading to much lower Myc levels than in AP border and posterior cells, suggesting that the CORD region has an additional role to inhibit Ci processing in the absence of Cos2 binding (Fig. 1.10A, blue arrows) (Zhou and Kalderon, 2010). The N-terminal region of Ci contains a Sufu binding site (green arrow, aa 212-268) and is important to moderate Ci activity.

However, the results from a *hh-lacZ* repression assay suggested that endogenous Cos2 can process Ci ΔCDNΔCORD (Methot and Basler, 2000; Zhou and Kalderon, 2010) (Fig. 1.10B). This assay is a sensitive measure for the generation of Ci-75 repressor. The

method takes advantage of the ability of Ci-75 to repress the transcription of *hh*. Hh signaling in the P-compartment, a region of *hh* expression, is blocked by generation of *smo* clones (marked by the absence of GFP), allowing Cos2-mediated proteolysis. Because endogenous Ci is absent in the posterior wing disc (Held, 2002), it is possible to test the proteolysis of exogenous Ci products (Fig. 1.10B) such as the Ci ΔCDNΔCORD. If proteolysis occurs, the generation of Ci-75 reduces β-galactosidase (*βgal*) expression from the *hh-lacZ* reporter construct (red). This method is a sensitive measure of Ci processing, because certain Ci variants that show a processing defect in the anterior by Myc assay, such as Ci G3A, a Ci variant with a mutation in a GSK3 phosphorylation site (Smelkinson et al., 2007), can still repress *hh-lacZ* in the posterior *smo* clones. High sensitivity to Ci-75 makes *hh-lacZ* repression assay a convenient test for severe processing defects that result in a block of *hh-lacZ* repression.

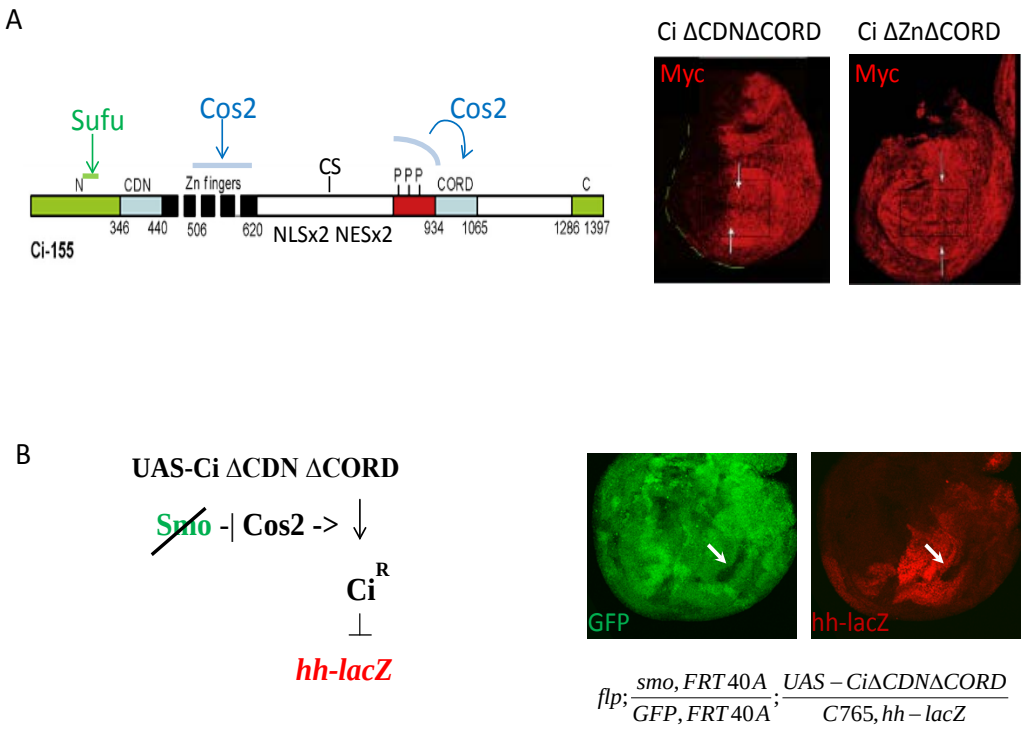


Figure 1.10. Topology of Ci and its Interactions with Cos2 and Su(fu) (taken from Zhou and Kalderon, 2010). (A) Diagram of Ci indicating the regions that are relevant for discussion of protein interactions (N, C (green); CDN, CORD (blue)) as well as the binding sites for Su(fu) and Cos2 (arrows and lines above the diagram). Deleting the CORD region supports the rescue of Ci-Myc elevation by Cos2 variant selectively deficient in binding to CORD (Cos2-S182N), suggesting that CORD region may play an additional processing-inhibitory role that is opposed by the binding to Cos2 (curved blue arrow). CS, cleavage site (aa 650-700); PPP, phosphorylation cluster (aa 838-895). On the right, endogenous Cos2 in the anterior (black) can reduce the level of Myc-Ci (red) without CORD and previously identified Cos2 binding site CDN (Wang and Jiang, 2004), but not without CORD and Zn finger region (Zn), suggesting that Zn fingers and not CDN provide the redundant binding site for Cos2. (B) Schematic representation of *hh-lacZ* assay used to demonstrate that CiR is still generated from CiΔCDNΔCORD transgene. First, *smo* clones (marked by the absence of GFP in the first panel on the right) are generated in the posterior compartment (marked by the expression of *hh*-lacZ (red) in the second panel on the right) of a disc wing disc that ubiquitously expresses CiΔCDNΔCORD, which initiates processing by endogenous Cos2. The ability of Cos2 to process CiΔCDNΔCORD is evaluated by generation of CiR that represses the production of β-galactosidase (βgal, red) from *hh*-lacZ reporter.

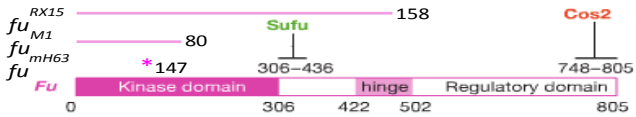
Cos2 S182N, a variant of Cos2 that selectively cannot bind to CORD, facilitated the discovery of the processing-inhibitory role of CORD in the absence of Cos2 binding (Zhou and Kalderon, 2011). The S182N alteration targets the ability of Cos2 to bind and hydrolyze ATP and impairs Cos2 motility (Ho et al., 2005; Farzan et al., 2008). GST binding assays demonstrated that ATP potentiates the interaction between wild-type Cos2 and CORD and Cos2 S182N fails to associate with GST-Ci-CORD. Cos2 S182N was highly defective in supporting processing of wild-type Ci, as can be seen from high Myc-tagged Ci levels in *cos2* mutant clones with over-expressed Cos2 S182N. However, Cos2 S182N supported processing of Ci lacking the CORD region much more efficiently. Hence, it appears that some part of the CORD region (not necessarily sequences that bind to Cos2) normally inhibits processing and this inhibition is prevented by binding of Cos2 to the CORD domain.

Fu Variants and Su(fu)^{LP}

X-ray and DEB (Diepoxibutane) mutagenesis screens uncovered 18 viable *fu* alleles that produced a fused-wing phenotype (the apparent fusion of veins 3 and 4 in the adult wing due to loss of v3-v4 inter-vein territory, Fig. 1.4). These alleles were assigned to two classes depending on their semidominant phenotypic supression by *Su(fu)^{LP}* (Therond et al., 1996). *Su(fu)^{LP}* is a null mutation that produces no phenotype on its own, but can partially rescue the fused wing defect in class I mutants like *fu^{mH63}* when present in even one copy (Pham et al., 1995). However, class II alleles additionally produce ectopic activation of the Hh pathway in a homozygous *Su(fu)^{LP}* background. Ci processing still occurs in the absence of Su(fu) alone, although both Ci-155 and Ci-75 levels are much

lower than normal, reflecting a role for Su(fu) in stabilizing both proteins. Importantly, Ci processing is impaired for class II alleles (like fu^{M1}, fu^{A} and fu^{94}) but not for class I alleles (like fu^{mH63} or fu^{1}) and this deficit likely accounts for ectopic induction of Hh target genes only for class II fu mutants in the absence of Su(fu) (the fu mutation elevates Ci-155 levels, while loss of Su(fu) removes an inhibitor of Ci-155 activity). (Methot and Basler, 2000; Lefers et al., 2001).

The molecular characterization of fu alleles showed that this functional classification is inherent in the domain structure of the Fu protein (Table 1.1). While class I alleles contain point mutations and small deletions in the Fu kinase region (Fu-kin, aa 0-306), class II alleles are generated by small deletions or nonsense mutations that remove large portions of the Fu regulatory domain (Fu-reg, aa 306-805) as a consequence of frame-shift mutations (Therond et al., 1996; Aikin et al., 2008).



	allele	synonim	structure
Class I	fu^{1}	-	* kin
	-	fu^{mH63}	* 147
Class II	fu^{54}	fu^{M1}	80
	fu^{55}	fu^{MC2}	158
	fu^{94}	-	Δ reg
	-	fu^{RX15}	474
	fu^{57}	fu^{W3}	612
	-	fu^{RX16}	638
	-	fu^{RX2}	748

Table 1.1. Structure of Fu Alleles

Class I alleles contain point mutations (*) in Fu-kinase domain (kin) and have no effect on Ci processing. *Class II* alleles contain truncations (Δ) in Fu-regulatory domain (reg, white bar) that account for their lengths

in amino acids, indicated by numbers in the "structure" column. Pink lines indicate the extent of two representative *class II* alleles, while pink star shows the location of the point mutation in fu^{mH63} (*class I*) allele.

Cos2 and Fu

IP of Myc-tagged Cos2 deletion variants from S2 cells followed by antibody analysis for the presence of Fu identified the smallest region of Cos2 (aa 543-605) sufficient for binding Fu (Ruel et al., 2007). This Fu-binding domain of Cos2 contains Ser572, one of the Hh-dependent Cos2 phosphorylation sites (Fig. 1.8).

IP of Fu from imaginal discs expressing the fu^{RX2} allele followed by blotting with antibody to Cos2 showed the loss of association between Fu and Cos2 (Table 1.1). Because the fu^{RX2} allele contains a C-terminal truncation that removes 57 amino acids of Fu regulatory domain, the carboxyl terminus of Fu was implicated in binding to Cos2 (Robbins et al., 1997). The regulatory domain of Fu was further implicated in the regulation of Ci processing because *fu* class II alleles that carry truncations of C-terminal domain showed ectopic accumulation of Ci in the anterior compartment (Alves et al., 1998; Lefers et al., 2001) and loss of *hh-lacZ* repression in the posterior compartment (Methot and Basler, 2000).

The loss of both Cos2 association and efficient Ci processing for Fu lacking the last 57 amino acids suggests that Cos2-Fu association is likely important for Ci processing. Fu-Cos2 association also likely affects the stability of both proteins. Fu levels are reduced in *cos2* clones (Lum et al., 2003b) and treating S2 cells with RNAi for Cos2 noticeably reduced the level of endogenous Fu (Ruel et al., 2003). Vice versa, RNAi for Fu reduced

the levels of Cos2 in S2 cells, suggesting that Cos2 may rely on Fu for stability (Liu et al., 2007; Aikin et al., 2008).

Genes for Fu and Cos2 were isolated in zebrafish, where Hh signaling controls the differentiation of slow-twitch muscle fibers (Wolff et al., 2003; Tay et al., 2005). In-situ hybridization analysis of zebrafish embryos demonstrated that both proteins are transcribed ubiquitously at low levels that did not change in response to mRNA against Shh. The studies of protein expression were limited by the lack of appropriate antibodies. However, reducing the expression of Cos2 by antisense morpholino oligonucleotides (MOs) resulted in ectopic activation of Hh signaling pathway from the expansion of *ptc1* expression domain (Tay et al., 2005), while the MO knock-down of *fu* decreased the expression of *ptc1* (Wolff et al., 2003). These findings suggest that, as in Drosophila, Cos2 is a negative regulator of Hh signaling in zebrafish and Fu is required for pathway activation.

Part IV. Ci activation and the Role of Cos2 in Response to Hh

Expression of distinct transcription targets (*ptc* and hh-dependent anterior engrailed (A-*en*)) in the region of highest Hh concentration (a stripe of 4-5 cells adjacent to the AP boundary) marks the highest level of Ci activity (Motzny and Holmgren, 1995; Wendler et al., 2006). Whether maximal Ci activation involves generation of a distinct form of the Ci activator or simply increased nuclear concentration, association with co-activators or dissociation of inhibitors is not known.

Hh Increases the Proportion of Ci in the Nucleus

A direct way of looking at the effect of Hh on Ci nuclear trafficking in vivo is to stain the wing disc with Ci antibody that recognizes the full-length form of Ci but not Ci-75 and to compare the sub-cellular distribution of Ci in Hh-receiving cells to the signal from more anterior cells using high-magnification confocal microscopy. Early studies that used this method detected cytoplasmic accumulation of Ci but failed to detect any Ci staining in the nuclei throughout the anterior compartment (Sisson et al., 1997). In a more sensitive assay, western blot analysis of Cl8 cell extracts detected Ci in both cytoplasmic and nuclear fractions of Hh-treated cells, but Ci was absent from the nuclear fractions of Hh-untreated cells (Chen et al., 1999). The results of the fractionation experiment were replicated by immunofluorescence staining but were much clearer if tissue culture cells or wing discs were pre-treated with leptomycin B (LMB), an inhibitor of CRM1 receptor for nuclear export. Indeed, nuclear Ci was readily detected at much higher levels in Hh-responding cells than in the rest of the anterior cells of the wing discs that were treated with LMB (Wang et al., 2000). Also, *smo* mutant clones that block Hh signaling do not show nuclear Ci accumulation after LMB treatment (Wang and Holmgren, 2000). Thus, Hh regulates nuclear accumulation of Ci; Ci accumulation is opposed by nuclear export, and the majority of Ci remains in the cytoplasm.

Anterior cells of LMB-treated wing discs accumulate much higher levels of nuclear Ci in *cos2* mutant clones, suggesting that association of Cos2 with Ci retains Ci in the cytoplasm (Wang et al., 2000). In a more artificial assay using tagged Ci variants, excess Cos2 was able to retain Ci in the cytoplasm of LMB-treated tissue culture cells but only if

Ci retained the CORD domain, suggesting that binding of Cos2 to CORD can suffice (under these non-physiological conditions) for cytoplasmic retention by Cos2.

Another protein that has a role in cytoplasmic retention of Ci is Su(fu) (Ohlmeyer and Kalderon, 1998; Wang and Holmgren, 2000). Studying the role of Su(fu) in Ci trafficking is complicated by the effect of Su(fu) on Ci stability. Immunofluorescence and western blot analyses show that Ci levels are reduced in *Su(fu)* mutant cells. Nuclear translocation experiments in *Su(fu)* mutant tissue were therefore conducted under Ci-over-expressing conditions with stabilized Ci (Ci^{PKA4}) and salivary glands were used for their enlarged nuclei. Under these conditions, Ci was retained in the cytoplasm of the wild-type salivary glands and accumulated in the nucleus of *Su(fu)* mutant glands (Methot and Basler, 1999). Also, Sufu over-expression caused Ci to be retained in the cytoplasm independent of LMB treatment. In the same set of experiments, immune fluorescence analysis did not detect accumulation of Su(fu) in the nucleus under both LMB-treated and untreated conditions.

Fu kinase opposes the effect of Sufu on Ci (Therond et al., 1996; Ohlmeyer and Kalderon, 1998; Wang et al., 2000). A point mutation in Fu kinase domain, fu^{mH63}, blocks the kinase activity of Fu. Cells at the AP border of fu^{mH63} wing discs treated with LMB do not accumulate nuclear Ci in response to Hh compared to Hh-receiving cells of the wild type wing discs. Also, the fu^{mH63} mutation has an opposite effect to Su(fu) on the level of Ci, greatly increasing Ci-155 levels at the AP border, presumably because Cul3/Hib degradation is prevented by limiting Ci activation (Ohlmeyer and Kalderon, 1998; Zhang et al., 2006; Zhou and Kalderon, 2011). Finally, Fu kinase activity opposes the effect of Su(fu) on the expression of Hh transcriptional targets which pattern the region between

wing veins 3 and 4, because fusion of veins 3 and 4 in fu^{mH63} mutant discs is suppressed by the removal of Su(fu). Fu kinase was proposed to antagonize cytoplasmic retention of Ci by Su(fu) (Ohlmeyer and Kalderon, 1998).

The transcriptional response to high levels of Hh (A-*en*) is lost in fu^{mH63} clones and *cos2* clones (Wang et al., 2000). Fu kinase and Cos2 are therefore essential for a normal response to Hh. However, little is known about their individual roles in promoting Ci activation or the role of activated Smo in transmitting the Hh signal to these cytoplasmic proteins. I will first describe what is known about the role of Smo in response to Hh then discuss the focus of this study, what is known about the role of Fu and Cos2.

Smo Activity Depends on Phosphorylation of the Smo C-terminal Cytoplasmic Tail

Western blot analyses, mobility shift assays and in-vitro immunostaining showed that Hh induces Smo phosphorylation and membrane accumulation (Alcedo et al., 2000; Denef et al., 2000; Shi et al., 2011). Mutational analysis of the Smo C-terminal cytoplasmic tail (C-tail) revealed a cluster of important consensus PKA phosphorylation sites that prime phosphorylation by CK1 and GSK3 (Jia et al., 2004; Zhang et al., 2004; Apionishev et al., 2005; Zhou et al., 2006). Replacing these residues with alanine (A) to prevent phosphorylation greatly reduced the expression of *ptc-lacZ* reporter and the activity of Smo (Apionishev et al., 2005; Zhao et al., 2007; Fan et al., 2012). Moreover, replacing the Serine (S) phosphorylation sites with phospho-mimetic aspartate (D) residues resulted in increased membrane localization of Smo in S2 cells, which mimics the response of Smo to Hh (Jia et al., 2004; Zhao et al., 2007; Fan et al., 2012). However, deleting the region of Smo that contains the phosphorylation clusters (aa 661-818) also increased the

cell surface expression of Smo. A closer examination of the sequence for Smo autoinhibitory domain (SAID) revealed that the phosphorylation clusters (S1-S3) within this region are adjacent to arginine (Arg) motifs (R1-R4) (Zhao et al., 2007). Mutating positively-charged Arg clusters to Ala increased the membrane accumulation of Smo and induced ectopic *ptc-lacZ* expression in the wing disc, copying the effect of phospho-mimetic mutations. Phosphorylation of SAID on Smo C-tail promotes Smo activation and cell surface accumulation by neutralizing the positive charges of Arg motifs.

Hh-Dependent Phosphorylation Changes the Conformation of Smo C-tail

Fluorescence resonance energy transfer (FRET) is a biochemical assay that detects the decrease in distance between two molecules as an increase in energy transfer between yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) (Centonze et al., 2003). This assay was used to detect the conformational change associated with Smo activation (Zhao et al., 2007). When the fluorescent tags were attached to the extracellular amino (N)-termini of Smo molecules, the FRET signal was high independent of Hh stimulation, suggesting that Smo forms constitutive dimers through its N-terminal domain (SmoN). Hh stimulation significantly increased the FRET signal from Smo molecules with tagged C-tails, apparently increasing the proximity of Smo C-tails within Smo dimer. The C-tail also adopted a more open conformation relative to the third intracellular loop (L3) of the same molecule of Smo, consistent with a drop in FRET of Hh-treated S2 cells transfected with Smo tagged at both the C-tail and L3 (in the same molecule). C-tails of phospho-deficient variants of Smo could not adopt an open conformation relative to L3, suggesting that Smo phosphorylation stimulates the change to an active conformation.

Clustering of Smo C-tails Activates Hh Transcription Targets

To test the physiological significance of Smo clustering for Hh transcription response, *Zhao et al.* developed an inducible dimerization system (Zhao et al., 2007). The dimerization system takes advantage of mammalian tyrosine kinase EphB2 that forms hetero-tetramers in response to ephrin B2 (EB2) ligand. EphB2/EB2 clustering of Smo C-tails occurred when chimaeric proteins consisting of Smo C-tail and EB2 were co-expressed with EphB2. Clustering of C-tails led to ectopic activation of high Hh response target genes *ptc* and *en* in wing discs that was prevented by using $SMOC^{SA}$, a phosphodeficient form of the Smo C-tail. The protein kinase Gprk2/GRK2 is a conserved regulator of Smo activation in flies and mammals that stimulates clustering of Smo C-tails (Ayers and Thérond, 2010).

Smo as a GPCR: Gprk2 Promotes Clustering of Smo C-tails

Smo is structurally related to G-protein coupled receptors (GPCRs) that signal through heterotrimeric G proteins (Ogden et al., 2008). RNAi experiments in Cl8 cells showed that the level of Hh-dependent activity reporter *ptc*-luciferase depends on the level of G protein α_i ($G\alpha_i$), although the activating effect of $G\alpha_i$ on Hh signaling was not detected in an earlier large-scale RNAi screen of Drosophila genes that used a similar experimental design (Lum et al., 2003a). $G\alpha_i$ typically inhibits the activity of adenylate cyclase, which converts ATP to cAMP (Kristiansen, 2004). Hh treatment decreased the concentration of cAMP in Cl8 cells (Ogden et al., 2008). Cells that were transfected with either smo or $G\alpha_i$ dsRNA failed to increase cAMP in response to Hh, suggesting that Hh increased the concentration of cAMP through Smo-dependent activation of $G\alpha_i$. Removing

$G\alpha_i$ also produced phenotypes in adult wings consistent with the disruption of Hh responses in the wing disc. However, the wing phenotype (size reduction) is not consistent with the loss of response to high levels of Hh, or the loss in *ptc* that was observed in $G\alpha_i$ clones (Ogden et al., 2008).

Although many loss-of-function alleles of $G\alpha_i$ were isolated and characterized in the past, they were not reported to have an effect on Hh signaling (Wolfgang et al., 2001). Instead, Ogden used $G\alpha_i^P$ alleles that were produced by P-element insertion into $G\alpha_i$ gene (Lineruth et al., 1992) and had general effects on animal viability and body architecture (Ogden et al., 2008). Further studies of the role of $G\alpha_i$ in Smo response to Hh would benefit from an allele that would specifically disrupt the interaction between Smo and $G\alpha_i$ (Ingham and McMahon, 2001).

GPCRs are directly phosphorylated by G-protein receptor kinases (GRKs) (Kristiansen, 2004). In-vitro kinase assays of GST-tagged Smo C-tail fragments identified Ser741/Thr742 as target sites for Drosophila G-protein receptor kinase 2 (Gprk2), and Gprk2 phosphorylation was promoted by PKA/CK1 phosphorylation of adjacent Ser residues (Chen et al., 2010). IP of Smo deletion variants with Gprk2 from S2 cells showed that deleting the SAID region in the Smo C-tail abolishes the interaction between Smo and Gprk2 (Chen et al., 2010). FRET assays demonstrated that Hh stimulation increases the proximity between Gprk2 and Smo and promotes Gprk2 dimerization. Gprk2 also stimulates co-precipitation of Flag-tagged and HA-tagged phosphomimetic forms of the Smo C-tail. It was therefore concluded that binding of Gprk2 to the Smo C-tail in its active conformation and dimerization of Gprk2 molecules promotes dimerization of Smo C-tails (Chen et al., 2010). Gprk2 activity is required for high-level Hh signaling from activated

Smo, because ectopic *en* from constitutively active Smo (SmoD123) is lost in *Gprk2* mutant discs (Molnar et al., 2007; Cheng et al., 2012).

Hh Regulates Smo Activity through the Fu-Cos2 Protein Complex

Deletion of the Smo C-tail (aa 555-1035) prevented co-precipitation of a Myc-tagged Smo construct with Fu and Cos2 in S2 cells and wing disc extracts (Jia et al., 2003). Comparing band intensities from IPs with antibodies for endogenous Cos2 and Fu in S2 and Cl8 cells showed that Cos2 and Fu associate at a constant ratio in a Fu-Cos2 protein complex (Lum et al., 2003b). *Jia et al.* created a series of deletion variants of the Smo C-tail with Myr tags for membrane anchorage. Deletion analysis by IP of Smo C-tail (CT) variants with endogenous Cos2/Fu complex in S2 cells restricted the binding domain to the region of Smo C-tail between amino acids 818 and 1035. The Myr-SmoCT deletion variants were tested for the ability to activate Hh transcription targets in the wing disc. Overexpressing Myr-SmoCT constructs in the wing disc caused ectopic activation of *dpp-lacZ* reporter of Hh activity conditional on the presence of amino acids 730 to 1035. Therefore, the region of the Smo C-tail between amino acids 730 and 1035 was deduced to associate with the Cos2/Fu complex and to be essential for the activity of Smo (Jia et al., 2003; Lum et al, 2003b).

High Hh Signaling Activity Stabilizes the Association between Smo and Cos2

Deletion analysis of a series of Cos2 variants in S2 cells identified a direct binding site between Cos2 and Smo (Jia et al., 2003; Ruel et al., 2003). The Cos2 cargo domain associated with the Smo C-tail through Smo amino acids 900-1201. Hh stimulation of Cl8

cells stabilized the association between Smo and Cos2, leading to accumulation of Smo-Cos2/Fu complexes. Immune fluorescence analysis of embyos also showed increased colocalization of Smo and Cos2 in Hh-responding cells. Fu potentiated the interaction between Smo and Cos2 as HA-Cos2 pulled down more Smo from S2 cells when co-expressed with Fu. Phosphoamino acid analysis of radiolabeled Cos2 co-transfected with Fu into S2 cells using baculovirus expression system identified two Fu-dependent phosphorylation sites on Cos2: serine 572 (Ser572) and serine 931 (Ser931) (Nybakken et al., 2002). Immune fluorescence analysis with phospho-specific antibodies in the wing disc showed that, while Ser572 phosphorylation is characteristic of all Hh-responding cells, Ser931 phosphorylation is only present in the region of high Hh signaling that expresses *ptc* and *A-en* (Ranieri et al., 2012). IP of phospho-mimetic forms of Cos2 from Cl8 cells demonstrated that Ser572 phosphorylation weakens association between Smo and Cos2, while Ser931 phosphorylation stimulates the association between Smo and Cos2. Finally, the EphB2/EB2 inducible dimerization system was used to show that phosphorylation of Ser931 in the wing disc is conditional on clustering of Smo C-tails, establishing Ser931 phosphorylation as a marker of high levels of Hh signaling activity.

Cos2 is Required for Fu Dimerization and Activation

A point mutation that disrupts the kinase activity of Fu, fu^{mH63}, causes a loss of expression of anterior En and severe reduction of the level of a *ptc* reporter gene, showing that Fu kinase activity is required for the response to high levels of Hh. IP of a series of Fu truncation alleles from wing discs localized the region of Fu interaction with Cos2 to the last 57 amino acids in the regulatory domain of Fu (Table 1). Smo requires Cos2 to

activate Fu, which is supported by *smo cos2* clones that fail to induce the expression of anterior En in the presence of activated Smo (SmoD123) (Zhou and Kalderon, 2011). Anchoring Fu at the membrane through a palmitoylated GAP43 domain results in strong Hh pathway activation, because overexpression of GAP-Fu protein produces ectopic En in the anterior compartment (Claret et al., 2007). Immune fluorescence analysis showed that Hh stimulation induces vesicle-to-membrane transclocation of Fu in Cl8 cells that depends on physical association between Smo and Fu (Claret et al., 2007; Malpel et al., 2007). Moreover, high resolution images of wing discs with fu clones demonstrated that Fu is required for Smo accumulation (Claret et al., 2007). Band shift analysis of western blots from S2 cell extracts showed that cotransfection with GFP-Fu promoted Smo phosphorylation, which was observed in larger amounts if GAP-Fu variant was used instead of GFP-Fu. It was argued that Fu activation depends on membrane localization and is necessary to activate Smo (Claret et al., 2007).

A series of FRET experiments in combination with transcriptional reporter assays established that Fu dimerization is another component of Fu activation, and that Fu dimerization depends on Cos2 (Shi et al., 2011; Zhang et al., 2011). FRET efficiency between CFP-Fu and YFP-Fu transfected in equal amounts into S2 cells increased in response to the increase of Hh concentration in the Hh-conditioned medium, suggesting that Hh stimulates the clustering of Fu molecules in a dose-dependent manner (Shi et al., 2011). Importantly, RNAi for Cos2 prevented the increase in FRET efficiency in S2 cells that coexpress CFP-Fu and YFP-Fu in response to Hh, suggesting that Cos2 is required for Fu clustering (Zhang et al., 2011). To demonstrate the effect of Fu dimerization on the activation of Hh transcription targets, *Shi et al.* constructed a chimeric variant of Fu with

dimerization motif from yeast GCN4 at the N-terminus (CC-Fu) and expressed the transgene in the dorsal part of the wing disc using *ap-Gal4* driver. Expression of CC-Fu induced ectopic expression of *dpp-lacZ* reporter and Ptc in the dorsal compartment of the wing disc (Shi et al., 2011).

Phosphorylation of Fu is Essential for the Activity of Fu

Like many kinases, Fu is capable of autophosphorylation (Malpel et al., 2007; Pike et al., 2008). Sequence analysis of Fu protein kinase revealed a conserved phosphorylation loop substrate for phosphorylation-dependent regulation of kinase activity (Zhou and Kalderon, 2011). The activation loop (AL) of Fu kinase domain contains the likely site of Fu autophosphorylation, Ser159 (Fukumoto et al., 2001; Malpel et al., 2007; Aikin et al., 2008). Replacing Ser159 with Ala produced a variant of Fu (Fu-S159A) which had a dominant-negative effect on pathway activity. Ubiquitous expression of Fu-S159A in the wing disc severely reduced the level of Ptc at the AP border, suggesting that S159 residue is essential for the activity of Fu (Zhou and Kalderon, 2011).

Zhou and Kalderon confirmed that Ser159 is the site of Fu trans-phosphorylation in an elegant in-vitro phosphorylation assay. The assay was conducted in KC cells that co-expressed Flag-Fu and HA-FuEE, where FuEE is a consistutively active form of Fu that produces robust gel mobility shifts on its own (Zhou and Kalderon, 2011). Indeed, Flag antibody detected a mobility shift of wild type Fu, but not kinase-defective Fu-S159A. However, even Fu-S159A shifted in response to Hh and activated Smo (SmoD123), pointing to the involvement of other phosphorylation sites in Fu activity (Shi et al., 2011; Zhou and Kalderon, 2011).

Band shift analyses of western blots from KC cell and wing disc extracts have demonstrated that Fu is extensively phosphorylated in response to Hh or activated Smo (Zhou and Kalderon, 2011). Band shifts from Fu phosphorylation in response to activated Smo were reduced by siRNA to casein kinase 1 (CK1), suggesting that CK1 phosphorylates Fu. Indeed, mutational analysis of Fu phosphorylation loop revealed functionally important CK1-dependent serine (S) and threonine (T) phosphorylation sites (Zhou and Kalderon, 2011). Fu variants with phosphomimetic (glutamic acid, E) or phosphodeficient (valine, V) substitutions of potential CK1 sites were expressed in the wing discs in the absence of Fu kinase activity and assayed for ability to rescue the expression of high Hh transcription targets at the AP border. Specifically, changing two of the candidate CK1 phosphorylation sites, T151 and T154, to acid residues rescued the expression of En and *ptc-lacZ* reporter (Zhou and Kalderon, 2011). Thus, CK1-dependent phosphorylation of Thr residues are required for Fu activity in addition to S159. Immunoblotting the extracts of S2 cells with phosphospecific antibodies against T151/T154 residues showed that the amount of phosphorylated Fu increases with the concentration of Hh in the conditioned medium, suggesting that Hh activity gradient is reflected in the gradient of Fu phosphorylation and activation (Shi et al., 2011; Chen and Jiang, 2013).

Fu Activity

Several key findings supported the idea that the principal role of Fu kinase in mediating Ci activation is to inhibit Su(fu) (Preat, 1992; Ohlmeyer and Kalderon, 1998; Methot and Basler, 1999; Methot and Basler, 2000). First, removing Su(fu) rescued the

fused wing phenotype in fu^{mH63} animals (Preat, 1992). Second, *Ohlmeyer and Kalderon* showed by western blot analysis of wing disc and embryonic extracts that $Su(fu)^{LP}$ and fu^{mH63} mutations have opposite effects on Ci concentration without changing the level of CiR. Wing disc immunostaining experiments confirmed that $Su(fu)^{LP}$ and fu^{mH63} have opposite effects on Ci concentration, and the effects were confined to the region of active Hh signaling, with $Su(fu)^{LP}$ markedly reducing the level of Ci at the AP border (Ohlmeyer and Kalderon, 1998). Ectopic activation of *ptc-lacZ* reporter due to the expression of a processing-resistant form of Ci, Ci^{PKA4}, in the wing disc was rescued by overexpressing Su(fu) (Methot and Basler, 2000). It was proposed that Fu kinase opposes the binding and stabilization of Ci by Su(fu), which limits the activity of Ci (Ohlmeyer and Kalderon, 1998; Methot and Basler, 2000).

Mutational analysis of Su(fu) for potential Fu-dependent phosphorylation sites identified two residues, S321 and S324, that were essential for Su(fu) mobility shift in KC cells expressing constitutively active FuEE (Zhou and Kalderon, 2011). However, a Su(fu) trangene with alanine substitutions of both residues complemented the function of wild-type Su(fu) in $Su(fu)^{LP}$ wing discs, rescuing the expression of *ptc-lacZ* and anterior En (Zhou and Kalderon, 2011). The finding that Fu-dependent phosphorylation sites on Su(fu) had no functional importance disagreed with the past expectations of Su(fu) antagonism as the key mechanism by which Fu regulates the activation of Ci (Lum et al., 2003b; Ho et al., 2005). The inconsistency was explained by re-examining the founding argument of the postulate, that removing Su(fu) rescued the fused wing phenotype in fu^{mH63} animals (Preat, 1992). A more careful analysis of the fu^{mH63} wing discs showed that, while rescuing *ptc-lacZ* expression, the loss of Su(fu) did not completely rescue the expression of anterior En.

However, the expression of anterior En was not reduced by removing Su(fu) alone. Fu may regulate Ci activation through targets other than Su(fu) (Zhou and Kalderon, 2011).

Mutagenesis experiments identified Ser572 as a Fu-dependent phosphorylation site on Cos2, and wing disc immunostaining with phospho-specific antibody against Ser572-P localized the region of Cos2-Ser572-P expression to the region of Ci stabilization (Raisin et al., 2010). *Zhou and Kalderon* expanded this finding to include the activated forms of Fu, GAP-Fu and Fu-EE, which stabilized Ci in smo clones within $Su(fu)^{LP}$ wing discs (Claret et al., 2007; Zhou and Kalderon, 2011). Ala substitution of Ser572 prevented Ci stabilization in *smo cos2* clones that coexpressed Fu-EE and Cos2-S572A, suggesting that Fu-dependent phosphorylation of Cos2 is essential for Ci stabilization mechanism exclusive of Smo and Su(fu) (Zhou and Kalderon, 2011). However, GAP-Fu could still induce the expression of anterior En in *slimb Su(fu)* mutant clones, suggesting that the role of Fu in stimulating Hh pathway activity is not limited to Su(fu) antagonism and Ci stabilization (Zhou and Kalderon, 2011).

Briscoe and Therond have identified ten open questions of Hh signaling, four of which are studied in Drosophila (Briscoe and Thérond, 2013). Two of the latter questions, which are not the subject of our studies, address the mechanisms by which Hh travels through the tissues and the mechanisms by which Ptc regulates Smo (Rohatgi and Scott, 2007; Thérond, 2012). The other two questions deal with 1) the composition of cytoplasmic transduction complex and its role in mediating Ci processing without Hh (addressed in Chapter III) and 2) the mechanisms by which Hh inhibits Ci processing through Smo (addressed in Chapter IV).

Chapter II

Materials and Methods

Mutagenesis and Cloning

A 6.5-kb fragment of genomic Cos2, extending from a KpnI site (position 131) to ORF frame in the plasmid vector pCaSpeR4 had been used to rescue Cos2 function in flies (Sisson et al., 1997) and supplied to us by Matt Scott. We introduced modifications in order to incorporate this fragment into an ATT vector, add an excisable transcription termination cassette in the first intron, and modify Cos2 coding sequence in a variety of ways, all by using oligonucleotide-mediated mutagenesis (QuikChange, Stratagene). First, an SphI restriction site was introduced in place of the KpnI site upstrteam of the Cos2 transcription unit. Next, an AvrII site was introduced near the beginning of *intron 1* sequence. Mutations of interest ΔFu (removes codons for amino acids (aa) 540-560 (Ruel et al., 2007)), S182N (Ho et al., 2005), S572A, S572D, S931A, S572A S931A (Ranieri et al., 2012) were then introduced into the modified Cos2 fragment. A larger deletion that removes codons for aa 540-600 (Ruel et al., 2007) was tried initially for ΔFu, but resulted in unstable protein and was abandoned. The following oligonucleotides were used for these site-directed mutageneses:

KpnI to SphI AGA CTC GAG GAA TTC *GCA TGC* GTT GTG AGT ACG GCG

AvrII in intron1 CCA GGT GAG TTT ACT *CCT AGG* ATC TTA TAA GCA TAG

ΔFu GAT CTG GAC GAC AAG ATA TAT CTA TCC AAG

S182N GCG CGG CCA AGG CAA *AAA* CTA CAC ACT CTA C

S572A AAG GCG GTT ATG CAA *GCC* CAA GAC CGC GAG ATT

S572D AAG GCG GTT ATG CAA *GAC* CAA GAC CGC GAG ATT

S931A ATC ACG GGC CAC CGC *GCC* ATC GAC ACG AGC GAC

S931D ATC ACG GGC CAC CGC *GAC* ATC GAC ACG AGC GAC

The restriction sites or amino acid modifications are in italics, and the underlined bases represent changes from the original sequence of the Cos2 fragment. An FRT-flanked transcription terminator cassette FC22 contained on a 3.5-kb NheI fragment was cloned into the AvrII site of each construct. The cassette was from Gary Struhl, Columbia University (Struhl and Basler, 1993); sequence information is provided in the next section; see also Fig. 2.1. The fragments were sub-cloned into the pUASattB vector (Bischof et al., 2007) between SphI and KpnI restriction sites and inserted at cytological locations 86F and 53B2 (Rainbow Transgenic Services, Inc.).

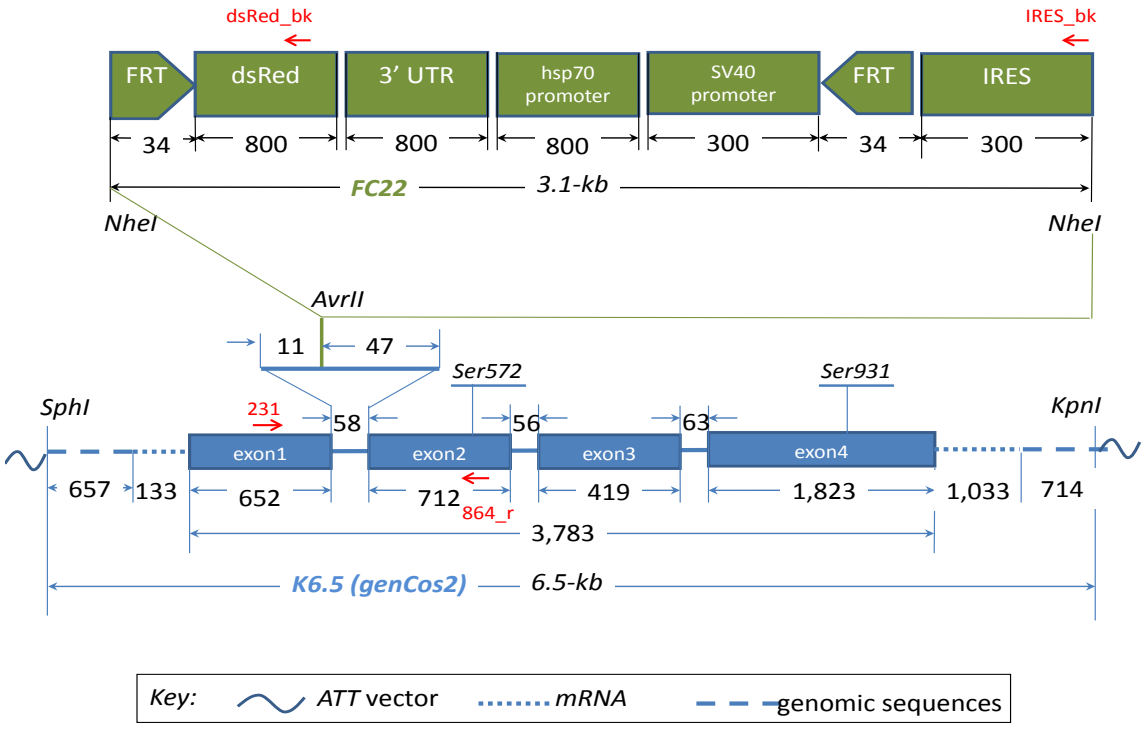


Figure 2.1. Maps of Components for Making pATT-gCos2 and How They are Assembled

Transcriptional terminator FC22 (green) is inserted into the AvrII site of gCos2 or gCos2 variant (blue) between the SphI and KpnI sites of ATT vector for site-specific integration on Drosophila chromosome. Numbers represent length in base pairs (bp) unless otherwise specified. The restriction sites that are used in cloning are shown along with the serine residues of Cos2 that are phosphorylated by Fu and targeted by the mutations of interest. Relevant primers are represented by red arrows and have the following sequences:

231: ACA ATT CCA GAT GCC ATT GG

864_r: CTG CAA CAG CCC CTC CTT GG

dsRed_bk: TGG AAC TGG GGG GAC AGG ATG

IRES_bk: GCC TTA TCC TCT AGT TGG TA

Gateway® technology was used to make a UAS-Cos2ΔFu construct for germline transformation and expression vectors for tissue culture tranfection (Invitrogen). The ΔFu mutation was introduced using site-directed mutagenesis into the coding sequence of Cos2 that had previously been cloned into pENTR/ D-TOPO vector (Marks and Kalderon, 2011) using the oligonucleotide shown above. The resulting entry vector was transferred to the pTW destination vector from the Drosophila Gateway® Vector collection by LR recombination (Invitrogen) to produce pUASt-Cos2ΔFu, which has N-terminal triple Flag tags and was introduced into the Drosophila germline by P-element transformation. Two lines on the 3rd chromosome, UAS-Cos2ΔFu(1) and UASCos2ΔFu(2) were used in functional tests. Drosophila Gateway® vectors pAHW, pAFW, pAMW and pAGW were used to make expression vectors for tissue culture transfection from the entry clones containing sequences for Cos2, Cos2ΔFu and Fu. The Cos2 proteins were tagged with Flag and Fu was tagged with HA at N-terminal ends.

Sequence of FC22 Transcriptional Termination Cassette

GCTAGCCCTAGAGGATCCCCGGGTACCTCGAGAAGTTCTTCTTCTCTAGCAAGAATAGGAACTTCGGAATA

GGAACTTACTAGTCGCCACCATGGCCTCCTCCGAGGACGTCATCAAGGAGTTCATGCGCTTCAAGGTGCGC

atgGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGC

ACCCAGACCGCCAAGCTGAAGGTGACCAAGGGCGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCCCA

GTTCCAGTACGGCTCCAAGGTGTACGTGAAGCACCCCGCCGACATCCCCGACTACAAGAAGCTGTCCTTCC

CCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACT

CCTCCCTGCAGGACGGCTCCTTCATCTACAAGGTGAAGTTCATCGGCGTGAACTTCCCCTCCGACGGCCCCG

TAATGCAGAAGAAGACTATGGGCTGGGAGGCCTCCACCGAGCGCCTGTACCCCCGCGACGGCGTGCTGAA

GGGCGAGATCCACAAGGCCCTGAAGCTGAAGGACGGCGGCCACTACCTGGTGGAGTTCAAGTCCATCTAC

ATGGCCAAGAAGCCCGTGCAGCTGCCCGGCTACTACTACGTGGACTCCAAGCTGGACATCACCTCCCACAA

CGAGGACTACACCATCGTGGAGCAGTACGAGCGCGCCGAGGGCCGCCACCACCTGTTCCTGTAGCGGCCG

GTGgagctcGCCCACTAAGCGTCGCGCCACTTCAACGCTCGATGGGAGCGTCATTGGTGGGCGGGGTAACC

GTCGAAATCAGTGTTTACGCTTCCAATCGCAACAAAAAATTCACTGCAACACTGAAAAGCATACGAAAACG

ATGAAGATTGTACGAGAAACCATAAAGTATTTTATCCACAAAGACACGTATAGCAGAAAAGCCAAGTTAAC

TCGGCGATAAGTTGTGTACACAAGAATAAAATCGGCCAGATTCAGTGTTGTCAGAAATAAGAAAACCCCAC

TATGTTTTTCTTTGCCTTTTCTTTCTCCCAGCGATCATTCATTTCGTGGTGAAAGAACGGGGTCATTGCACGG

AGTTTCGACTGCGGGAAAGCAGAGCTGCCGTTCACTTCGTCTATAATTAGCGCTTTCTATTTTCCCCGATTC

GGGCCGCTGCTGCGCTTTTCCGCCTGCTGTTTGTGGCAAGTGTAGCAGCAGGCTGTGCACGCAGTGTGGC

ATGCACTTGGCTTTCCACCGTTGGTATCGATTCTCTGGGACGATGAGTCATTCCTTTCGGGGCCACAGCATA

ATCGTTGCCAGCTCACCGAAATGGTGACTTCATTTCTTAACTGCCGTCAAGCATGCGATTGTACATACATAC

ATATTTATATATGTACATATTTATGTGACTATGGTAGGTCGATATAATAGCAATCAACGCAAGCAAATGTGT

CAGTCCTGCTTACAGGAACGATTCTATTTAGTAATTTTCGTTGTATAAAGTAATTATGTATGTATGTAAGCCC

CATAAATCTGAAACAATTAGGCAAAACCATGCGAAGCTCTCTAGTCGACTAAGGCCAAAGAGTCTAATTTT

TGTTCATCAATGGGTTATAACATATGGGTTATATTATAAGTTTGTTTTAAGTTTTTGAGACTGATAAGAATGT

TTCGATCGAATATTCCATAGAACAACAATAGTATTACCTAATTACCAAGTCTTAATTTAGCAAAAATGTTATT

GCTTATAGAAAAAATAAATTATTTATTTGAAATTTAAAGTCAACTTGTCATTTAATGTTTTGTAGACTTTTGA

AAGTCTTACGATACAATTAGTATCTAATATACATGGGTTCATTCTACATTCTATATTAGTGATGATTTCTTTA

GCTAGTAATACATTTTAATTATATTCGGCTTTGATGATTTTCTGATTTTTTCCGAACGGATTTTCGTAGACCCT

TTCGATCTCATAATGGCTCATTTTATTGCGATGGACGGTCAGGAGAGCTCCACTTTTGAATTTCTGTTCGCA

GACACCGCATTTGTAGCACATAGCCGGGACATCCGGTTTGGGGAGATTTTCCAGTCTCTGTTGCAATTGGT

TTTCGGGAATGCGTTGCAGGCGCATACGCTCTATATCCTCCGAACGGCGCTGGTTGACCCTAGCATTTACAT

AAGGATCAGCAGCAAAATTTGCCTCTACTTCATTGCCCGGAATCACAGCAATCAGATGTCCCTTTCGGTTAC

GATGGATATTCAGGTGCGAACCGCACACAAAGCTCTCGCCGCACACTCCACACTGATATGGTCGCTCGCCC

GTGTGGCGCCGCATATGGATCTTAAGGTCGTTGGACTGCACAAAGCTCTTGCTGCACATTTTGCAGGAGTA

CGGCCTTTGACCCGTGTGCAATCGCATGTGTCGCGCCAGCTTGTTCTGCGAAATAAACTTCTTGGAGCAGA

TCCTAGAGCGACTAAAGCCAAATAGAAAATTATTCAGTTCCTGGCTTAAGTTTTTAAAAGTGATATTATTTA

TTTGGTTGTAACCAACCAAAAGAATGTAAATAACTAATACATAATTATGTTAGTTTTAAGTTAGCAACAAAT

TGATTTTAGCTATATTAGCTACTTGGTTAATAAATAGAATATATTTATTTAAAGATAATTGCGTTTTTATTGTC

AGGGAGTGAGTTTGCTTAAAAACTCGTTTAGATCTGTCCTCGACGGATCCGGACGAAGTTCCTATTCCGAA

GTTCCTATTCTCTAGTAAGTATAGGAACGAGCTCACTAAGAGTTTCAAAATCAAAATTGAGGAATACCAACT

AGAGGATAAGGCTACTTAAGGATCAAAAAACACCAAGGAGACGAGATTTTCTACCAAATCGAGAGACGAG

GGGCAGGTTAATTTCGTCATTTTTGGCCAAGACAGCAAATAGAGGAACAGCAAAGCGAAAATCATTTTATA

CCTCACACAACAACTACACACTAACTAAGATTAGGCTACGCAACTGTACATTGTACTTAAGTGTTCAAAGTA

TATTTAGTTGCTAGC

Recombineering

Initial attempts to clone the transcriptional terminator (FC22) fragment into the first intron of the genomic Cos2 fragment in ATT plasmid were unsuccessful (generating deletions around the FRT sites of the FC22 fragment). I therefore tried an alternative cloning approach using recombineering (Fig. 2.2A) (Copeland et al., 2001; Muyrers et al., 2001; Sharan et al., 2009). Recombineering uses a set of genes from bacteriophage λ to catalyze homologous recombination between a linear DNA fragment (FC22) and a specific site on target DNA (first intron of genCos2). The reaction happens in a strain of E. Coli (SW102, NCI http://ncifrederick.cancer.gov/research/brb/recombineeringInformation.aspx) that provides all of the enzymatic components. SW102 strain expresses the phage recombination genes under control of temperature-sensitive promoter (*cl857*) that becomes active in response to a brief heat shock (15' at 42^0C). SW102 cells are routinely cultured at 32^0C and extended incubation at high temperature is lethal. The strain is electro-competent, but has to undergo a series of washes in ddH2O to remove salt inhibitors each time before electroporation (NCI). In preparation for the recombineering procedure, SW102 cells are electroporated with the target vector (pATT-genCos2; BioRad MicroPulser, 5 ms, 100-120 V). The second component of the recombineering experiment is a double-stranded linear insert, which accounts for the targeting specificity of the reaction and the recovery of the recombinant product.

To meet the requirements of the recombineering reaction, the linear fragment (FC22) must undergo two modifications (Fig. 2.2A, B). First, the fragment must include a selectable marker (Kan) that is not contained in the target vector. If the experimental strategy does not include a counter-selection step that would remove the marker after

recombination, the challenge is to choose the location of the marker. A good candidate region (e.g., IRES sequences within FC22) does not contain important functional elements. If the region does not contain appropriate restriction sites for cloning, the marker is introduced in a separate prior round of recombineering (Fig. 2.2B) while the fragment is inside the vector (pGEM). The second modification of the linear fragment introduces 50-bp of flanking sequences that are homologous to the sequences that delimit the site of integration in the target vector (intron1a and intron1b). Using 50-bp homologies introduces targeting specificity that does not depend on the availability of restriction sites. The sequences are added to 5' ends of 20-mer primers that prime the amplification of the linear insert. Product specificity is very important and can be enhanced by using 1ng of linearized template.

Recombination occurs when the purified PCR product (intron1a_FC22-KAN_intron1b) is electroporated into the bacterial cells that contain the target vector (SW102+pATT-genCos2) and are induced to express the recombination genes. Electroporation into uninduced cells is used as a control. Both reactions are plated on Kanamycin and incubated at 32° C overnight (Fig. 2.2C).

Recombination is a rare molecular event that affects only a fraction of target vectors for high-copy plasmids. Colony PCR of 14 colonies from an efficient recombineering reaction showed that each SW102 colony contained a mixture of target vector and the recombinant product (Fig 2.2B). Final steps to resolve the recombinant product consisted of 1) linearizing the mixture of plasmids from an SW102 colony, 2) re-ligating, 3) transforming into DH5α and 4) re-plating on Kanamycin. Colony PCR of 14 colonies from transforming 2 µl of the ligation mixture into DH5α shows that each colony

was now enriched for the recombinant product and 4 colonies showed no trace of the target vector.

The integrity of the recombinant genCos2 product was confirmed by restriction digest and by complete sequencing. The construct was injected into flies at cytological location 86F (Rainbow Transgenic Services, Inc.). Although the desired molecular product was obtained, the protein failed to rescue Ci elevation in *cos2* clones, presumably after FRT cassette excision, because *cos2* clones were generated by a more difficult recombination between FRTs on two different chromosomes (Fig. 2.2D, E). Without the insert, genCos2 construct complemented Ci processing in *cos2* clones (data not shown). The FC22-Kan insert could prevent the production of Cos2 if the transcriptional terminator was not excised by FRT recombination or if Kan and IRES elements of the modified transcriptional terminator that remain after excision impair the splicing of genCos2. Diploid tissue from larvae that were raised for the *cos2* complementation experiment (Fig. 2.2D) were tested by PCR and showed efficient excision between the FRTs of the modified FC22 cassette (Fig. 2.2E), leaving the alternative explanation of disrupted splicing. It is likely that the Kanamycin resistance gene (700 bp) introduced new alternative splice sites that are predicted, for example, by using GENSCAN bioinformatics software (MIT, http://genes.mit.edu/GENSCAN.html).

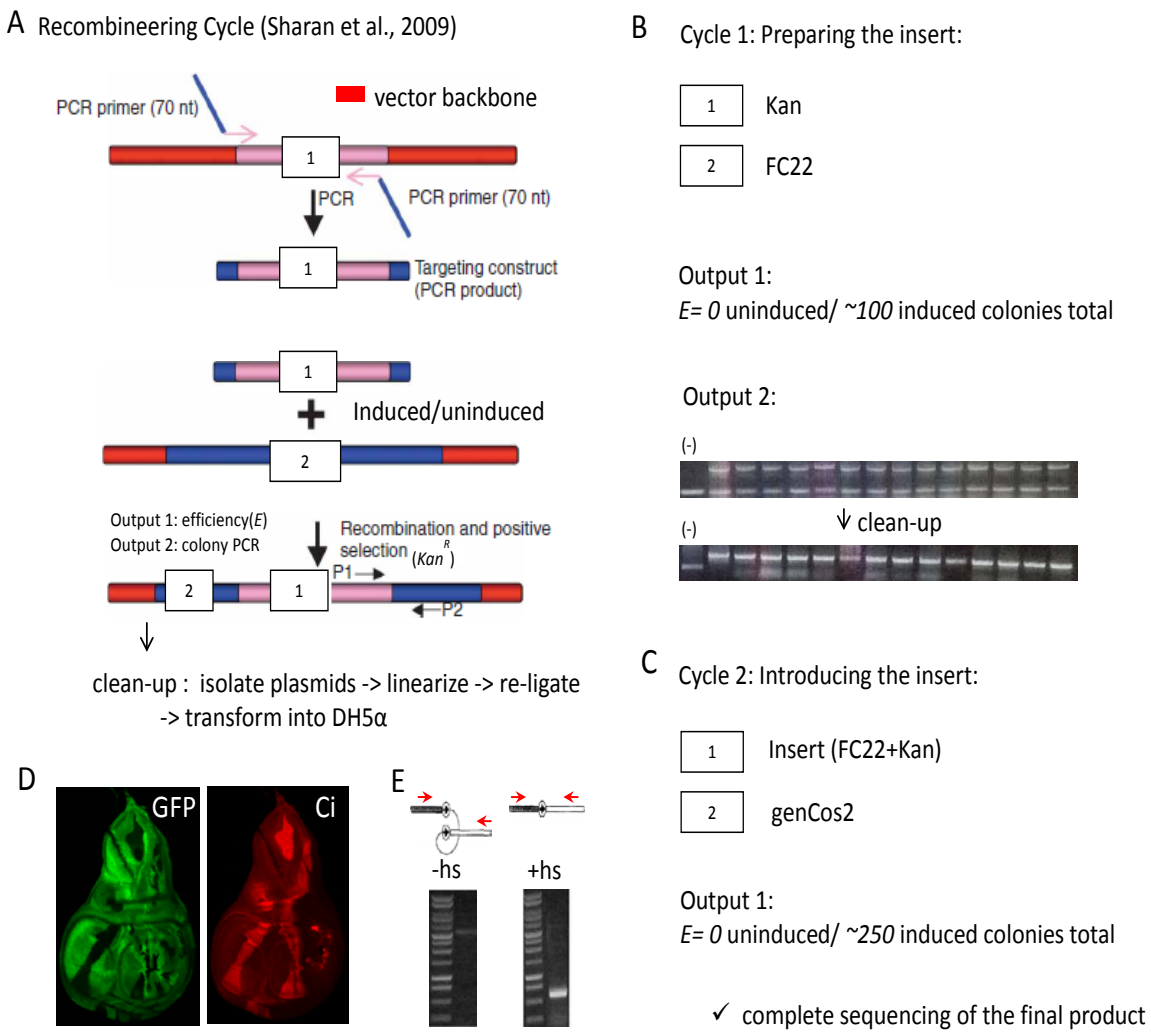


Figure 2.2. Insertion of Transcriptional Terminator FC22 into genCos2 by Recombineering (parts taken from Sharan et al., 2009).

(A) A typical recombineering procedure consists of PCR amplification of the insert (targeting construct) that contains a selectable marker (e.g. *Kan*, kanamycin-resistance gene) and 50 base flanking sequences that are homologous to the integration site in the target vector ('2'). The insert is electroporated into bacterial cells (e.g., SW102) that are induced to express recombination genes of phage λ or used as a negative control. The bacteria are grown in the presence of an appropriate antibiotic (positive selection for recombinant product) and the reaction efficiency is evaluated by comparing the number of colonies from induced and uninduced conditions (Output 1). (B, C) Two cycles of recombineering to be used with the diagram from (A): the first

cycle introduces selectable marker (Kan) into transcriptional terminator cassette (FC22); the second cycle inserts the cassette with the selectable marker into genomic Cos2 (genCos2). (B) PCR of SW102 colonies from induced condition with primers *231* and *IRES_bk* (that binds within the 50-bp region of homology shared by IRES and Kan) shows both IRES- (bottom) and KAN- containing product (top panel). After clean-up procedure from (A), PCR of DH5α colonies only detects FC22 plasmid with KAN only (bottom panel). First lane shows the product from original FC22 with IRES (negative control). (D) Immunostaining with 2A1 antibody (red) shows Ci elevation in GFP-negative *cos2* clones in the wing discs that ubiquitously express gCos2.(E) PCR of extracts from wing discs with gCos2 before (-hs) and after (+hs) heat shock. exon1 of genCos2, black bar; FC22: Kan instead of IRES, white bar; FRTs, circles; transcription terminator, thin line. Red arrows denote *primers 231 and IRES_bk*.

Insertion of Transcriptional Terminator FC22 into genCos2 by Recombineering

The insert was electroporated into SW102 bacterial cells that were induced to express recombination genes of phage λ or used as a negative control. The bacteria were grown in the presence of an appropriate antibiotic (positive selection for recombinant product) and the reaction efficiency (Output 1) was evaluated by comparing the number of colonies from induced and uninduced conditions (Fig. 2.2A). The colonies from the induced condition were further tested by colony PCR (Output 2). Plasmids from positive colonies underwent an additional clean-up procedure for high-copy plasmids to remove traces of the target vector. The recombineering procedure was used to introduce a selectable marker (‘1’=Kan) into the IRES sequence of the transcriptional terminator (‘2’=FC22 in pGEM vector) (Fig. 2.2B) and then to insert the transcriptional terminator that contains Kan (‘1’=FC22-Kan) into the first intron of the genomic Cos2 fragment (‘2’=genCos2 in ATT vector) (Fig. 2.2C).

Both cycles of recombineering were efficient, producing kanamycin-resistant colonies for induced condition and no colonies for uninduced control (Output 1, Fig. 2.2 B, C). Colony PCR of 14 potential FC22-Kan recombinants from cycle 1 (Output 2, Fig. 2.2A, B) showed that all colonies contain the target construct ('2'=pGEM-FC22; lower band) in addition to the desired product (pGEM-FC22-Kan; upper band), where the first lane represents SW102 colony with pGEM-FC22 (negative control). DNA from one of the FC22-Kan recombinants underwent a sequence of steps to eliminate the target construct (clean-up, Fig. 2.2A) and was transformed into DH5α. Colony PCR of DH5α colonies (lower panel) showed that 4 colonies contain only pGEM-FC22-Kan (upper band) and the remaining 10 colonies are enriched for pGEM-FC22-Kan with much weaker traces of pGEM-FC22 (lane 1, negative control).

Purified final product (pATT-genCos2-FC22-Kan) was checked by restriction digest and by complete sequencing of the genCos2 region (Fig. 2C). Absence of GFP (green) marks *cos2* clones in a wing disc that contains genCos2-FC22-Kan. The heat-shock used to induce *flippase* expression and produce *cos2* mutant clones is expected to eliminate the transcription terminator cassette also through recombination between the flanking FRT sequences. genCos2-FC22-Kan does not rescue Ci elevation in *cos2* clones. DNA was extracted from several wing discs of the same genotype from larvae that were subjected to heat shock (Fig. 2.2D). PCR of wing disc DNA template with primers *231* and *IRES_bk* (Figs. 2.1, 2.2E) produced a ~3.5-kb band without heat shock and ~700 bp product with heat shock, consistent with an excision event that removes 3-kb transcription terminator (Fig. 2.2E).

The terminator sequence was successfully introduced into the first intron of genCos2 by using conventional cloning methods after the standard QIAquick gel extraction kit was replaced with QIAEX II gel extraction kit (Qiagen), producing g>term>Cos2. The gel extraction step is necessary for isolation of FC22 fragment, and QIAEX II kit provides significant advantages over QIAquick kit by using a suspension of silica particles instead of a precipitation column, which allows for a greater manual control over the product purity and concentration. The kit was acquired for isolation of very large DNA fragments for a different cloning project, but could be used for a variety of DNA sizes and was tested on FC22. Reducing the final elution volume of ddH2O from 20 to 10 µl and using a hand-made glass pipette to extract the DNA solution without carrying over the beads produced highly concentrated (>100 ng/µl) and pure ($A_{260/280}$ > 1.8) FC22 fragment that consistently gave 1-2 positive clones in correct orientation in 18 colonies after ligation into pATT-genCos2 or genCos2 variant that were identified using colony PCR with primers *231* and *IRES_bk* (binds within the 50-bp homology region shared by IRES and KAN, Fig. 2.1).

Germline Excision of Transcriptional Terminator and Genotyping

Animals of genotype *yw hs-flp; smo^2 42D cos^{2}2 / PyCyO; (g>term>Cos2-WT, S572A, S572D, S931A, S572AS931A, S182N* and *ΔFu*) / *TM6B* were given a heat shock (37◦C, 1 h.) during the late third instar stage. Adult female progeny were collected 7 days later, and crossed to *yw hs-flp; 42D cos2^2 / PyCyO* males. The cross was transferred to a new vial after three days. After 7 days, the vials were inspected for *yw hs-flp; smo^2 42D cos^{2}2 /42D cos2^2* progeny rescued by a g>term>Cos2 excision event that produces gCos2.

Whole-animal viability rescue occurred with gCos2-WT, S572A, S931A, S572D, S931A, S572AS931A, but not with gCos2 S182N or gCos2-ΔFu proteins.

Animals of genotype *yw hs-flp; Sp / PyCyO; (g>term>Cos2-WT, S572A, S572D, S931A, S572AS931A, S182N* and *ΔFu*) / *TM6B* were given a heat shock (37◦C, 1 h.) during the late third instar stage. Adult female progeny were collected 7 days later, and crossed to balancer males. The cross was transferred to a new vial after three days. In 7 days, 5-10 adult male progeny were collected and crossed individually to balancer females. After 2 days, genomic DNA was harvested from each male and genomic PCR was used to confirm an excision event using two sets of primers: 1) *231* and *864_r* and 2) *231* and *dsRed_bk* (Fig. 2.1):

231: ACA ATT CCA GAT GCC ATT GG

864_r: CTG CAA CAG CCC CTC CTT GG

dsRed_bk: TGG AAC TGG GGG GAC AGG ATG

Progeny of males that tested positive for excision were used to make stocks. None of the Cos2 transgenes were lethal after excision and so experiments were performed with these derivatives (referred to as gCos2-WT, etc.) rather than the transgenes with transcriptional terminator cassettes (g>term>Cos2-WT, etc.).

Biochemistry (Cell culture, transfection, immunoprecipitation and western blot analysis)

Kc cells were kept at 25° C in Schneider's Drosophila media +5% FBS +1% Penicillin-Streptomycin (Gibco). Three 10 cm plates were seeded with $1x10^7$ cells and were given fresh media after 24 hours. The cells were transfected 3-4 hours later with 8

micrograms of Actin-HA-Fu together with Actin-Flag-Cos2, Actin-HA-Fu or Actin-Flag-Cos2ΔFu (8 μg each) using a calcium phosphate protocol (Invitrogen). Cells were given fresh medium 24 hours later and were harvested after 48 more hours. The cells were lysed at 4° C into 1 ml lysis buffer (50 mM Hepes (pH 7.5), 1.25 mM EDTA, 50 mM NaF, 0.5 mM $NaVO_3$ 0.5% NP-40, 1 mM DTT, protease inhibitors [mini complete, Roche]). The lysates were incubated with mouse anti-Flag antibody conjugated to agarose beads (Sigma) for 2 hours at 4^oC. The IPs were washed 3 times for 10 minutes in lysis buffer. The western blots were probed with mouse anti-Flag and rabbit anti-HA antibody (ab9110, Abcam). Secondary antibodies Alexaflour-680 and Alexaflour-800 were visualized with LI-COR Infrared imager, and the bands were quantified using LI-COR Odyssey Software.

In-vitro Immunostaining

S2 cells were kept at 25° C in Schneider's Drosophila media +5% FBS +1% Penicillin-Streptomycin (Gibco). Three 35 mm dishes with glass slides were seeded with $3x10^6$ cells and were given fresh media after 24 hours. The cells were transfected 3-4 hours later with 3 micrograms of Actin-Myc-Fu together with Actin-GFP-Cos2, Actin-Myc-Fu or Actin-GFP-Cos2ΔFu (3 μg each) using a calcium phosphate protocol (Invitrogen). Cells were given fresh medium 24 hours later and were allowed to express for 48 more hours. The slides were stained with mouse monoclonal 4A6 antibody to Myc (Millipore) using a standard immunohistochemistry procedure.

Immunohistochemistry

Clones were generated by heat-shocking 2nd instar larvae for 1-2 hours at 37° C. The animals were transferred to 29° C for high level of expression of UAS transgenes or were kept at 25° C. Late 3rd instar larvae were dissected into 4% paraformaldehyde on ice and rocked at 25° C for 30 minutes. Fixed samples were rinsed with 1X PBST (1X PBS +0.1% Triton X-100 +0.05% Tween 20) and placed in the blocking buffer (5% goat serum, 0.5% BSA, 50 mM Tris [pH 7.0], 150 mM NaCl, 0.5% BSA) for 2 hours at 4°C. The discs were stained with the following primary antibodies: rabbit anti-β-galactosidase antibody (Promega) for *lacZ* products, mouse monoclonal 4A6 antibody to Myc (Millipore), rat monoclonal 2A1 antibody to Ci (Motzny and Holmgren, 1995), mouse monoclonal 7E11 antibody to Cos2 and rabbit monoclonal antibody to Fu (Developmental Studies Hybridoma Bank, University of Iowa, USA). Secondary antibodies were anti-rabbit and anti-rat Alexaflour-594, anti-rabbit Alexafluor-488 and anti-mouse Alexafluor-680 (Molecular Probes). The discs were incubated with primary antibody for 1 hour at 25° C or at 4° C overnight. Then the samples were washed 3 times for 1 hour and incubated with the secondary antibody for 1 hour at 25° C. The samples were rinsed 2 times for 10 minutes in 1X PBST and the wing discs were mounted on glass slides in Aquapolymount (Polyscience). BioRad MRC-600 confocal microscope was used to examine the fluorescent staining. In Fig. 3.1, the areas within a rectangle were quantified and intensity plots were constructed using Image J software (NIH, Bethesda, MD). The x-axis is the width of the rectangle that represents the anterior-posterior width of the wing disc. The y-axis shows the average fluorescence intensity over the length of the rectangle at each point on the x-axis.

Fly Crosses

Females of the genotype *fu^{M1} / FM7* or *fu^{RX2} / FM7* were crossed to *yw hs-flp; +; C765 hh-lacZ / TM6B* males to generate *fu^{M1}* or *fu^{RX2} / Y; +; C765 ptc-lacZ /* + wing discs.

Females of the genotype *w fu^{M1}; P[Fu+]^{24B} FRT40A Sp / CyO; C765 hhlacZ / TM6B* were crossed to *yw; Sp / CyO; (UAS-Ci or UAS-CiΔCDNΔCORD) / TM6B* males to generate *w fu^{M1} / Y; (Sp or P[Fu+]^{24B} FRT40A Sp) / CyO* discs that express Ci or CiΔCDNΔCORD ubiquitously.

Females of the genotype *w fu^{M1}; P[Fu+]^{24B} FRT40A Sp / CyO; C765 hhlacZ / TM6B* were crossed to *yw; FRT40A ubi-GFP Flp^{38} / CyO; (TM2 or UAS-Cos2) / TM6B* males to generate *fu* mutant clones marked by two copies of ubi-GFP in male wing discs with or without expression of the Cos2 transgene.

Females of the genotype *w fu^{M1}; P[Fu+]^{24B} FRT40A Sp / CyO; (FRT82B Su(fu)^{LP}) C765 hhlacZ / TM6B* were crossed to *yw; smo^2 FRT40A Flp^{38} / CyO; UAS-Ci (Su(fu)^{LP}) / TM6B* males to generate *fu smo* mutant clones, detected by the absence of Fu staining, in male wing discs with or without Su(fu).

Females of the genotype *yw hs-flp, UAS-GFP; smo2 FRT42D tub-Gal80 hsCD2 P[Smo+] / CyO; C765 ptc-lacZ / TM6B* were crossed to *yw hs-flp; FRT42D $cos2^2$ / CyO; (UAS-Cos2 or UAS-Cos2ΔFu) / TM6B* males to generate positively marked *cos2* mutant clones expressing Cos2 or Cos2ΔFu.

Females of the genotype *yw hs-flp, UAS-GFP; smo2 FRT42D tub-Gal80 hsCD2 P[Smo+] / CyO; (FRT82B Su(fu)^{LP}) C765 hh-lacZ / TM6B* were crossed to *yw hs-flp; smo^2*

FRT42D cos2^2 / CyO; (UAS-Cos2 or UAS-Cos2ΔFu) (FRT82B Su(fu)^{LP})(UAS-Ci or UAS-CiΔCDNΔCORD)/ TM6B males to generate positively marked *smo cos2* mutant clones expressing UAS-Cos2 or UAS-Cos2ΔFu and UAS-Ci or UAS-CiΔCDNΔCORD with or without Su(fu).

Females of the genotype *yw flp; FRT42D P[y+] ubi-GFP / CyO; C765 ptc-lacZ / TM6B* were crossed to *yw hs-flp; smo^2 FRT42D cos2^2 (UAS-GAP-Fu) / CyO; TM2 or gCos2 (WT, S572A, S931A, S572AS931A, ΔFu, S182N) / TM6B* males to generate negatively marked *cos2* mutant clones in a wing disc expressing gCos2 (WT, S572A, S572D, S931A, S572AS931A, ΔFu, S182N) ubiquitously.

Females of the genotype *yw hs-flp, UAS-GFP; FRT42D tub-Gal80 hsCD2 P[Smo+] / CyO; C765 hh-lacZ / TM6B* were crossed to *yw hs-flp; smo^2 FRT42D cos2^2 (UAS-GAP-Fu or UAS-SmoD123) / CyO; TM2 or gCos2 (WT, S572A, S931A, S572AS931A, ΔFu, S182N) / TM6B* males to generate to generate positively marked *cos2* mutant clones expressing gCos2 (WT, S572A, S931A, S572AS931A, ΔFu, S182N) ubiquitously with or without UAS-GAP-Fu and for gCos2 (WT, ΔFu, S182N) with or without UAS-SmoD1-3.

Females of the genotype *yw hs-flp, UAS-GFP; smo2 FRT42D tub-Gal80 hsCD2 P[Smo+] / CyO; C765 hh-lacZ / TM6B* were crossed to *yw hs-flp; smo^2 FRT42D cos2^2 UAS-CiΔΔ/ CyO; TM2 or gCos2 (WT, ΔFu) or UAS-Cos2 / TM6B* males to generate to generate positively marked *smo cos2* mutant clones expressing gCos2 (WT, ΔFu) ubiquitously with UAS-CiΔΔ.

Chapter III

Results

Efficient Ci-155 Processing Requires Fu

Prior studies clearly show that the regulatory domain of Fused is required for efficient Ci-155 processing (Methot and Basler, 2000; Lefers et al., 2001). Moreover, incomplete evidence from the early studies of *fu* alleles suggests that there may be a correlation between the severity of truncation in the Fu regulatory domain and the extent of Ci-155 elevation in the anterior wing discs (Wang and Holmgren, 1999; Lefers et al., 2001). This trend is suggested by comparing the data for two alleles of *fu*, the longer fu^{RX15} allele, which encodes 474 of the 805 amino acids (aa) in wild type Fu, and the shorter fu^{94} allele, which codes for only about 351 aa in Fu protein, removing the majority of the Fu regulatory domain (Table 1.1). Wing discs homozygous for the fu^{RX15} allele showed increased Ci-155, detected by staining with 2A1 antibody in anterior cells beyond the normal range of Hh signaling (Wang and Holmgren, 1999). However, the anterior-most region of fu^{RX15} wing discs did not show a clear increase in the level of Ci-155 (Wang and Holmgren, 1999). In these wing discs *ptc* induction is greatly reduced and consequently Hh travels further into the anterior compartment (Chen and Struhl, 1998). The elevation of Ci-155 beyond the normal range of Hh was therefore interpreted as being caused principally by excessive spread of Hh (Wang and Holmgren, 1999). By contrast, Ci-155 levels were reported to be increased throughout the anterior compartment in wing discs homozygous for the fu^{94} allele (Lefers et al., 2001). Furthermore, Ci-155 levels were increased cell-autonomously in anterior fu^{94} mutant clones far from the AP border,

confirming that Fu is directly required for Ci-155 processing in anterior cells in the absence of Hh (Lefers et al., 2001). We wanted to check if there was indeed a reproducible difference in the pattern of anterior elevation of Ci-155 and the underlying mechanism among *fu* alleles encoding proteins of different length.

We began by looking at the pattern of Ci-155 staining in wing discs homozygous for three representative alleles of Fu – fu^{M1}, which encodes a very short protein (only 80 of the total 805 aa), the fu^{W3} allele (encoding 612 aa), and the fu^{RX2} allele (encoding 748 aa) that lacks the sequence for 57 C-terminal aa (Fig. 3.1A). Anterior cells away from the AP border normally mediate efficient Ci-155 processing and have clearly lower levels of Ci-155 than cells at the AP border in wild-type wing discs (Fig. 3.1B). For all *fu* alleles we examined (fu^{M1}, fu^{W3} and fu^{RX2}), we saw elevated Ci-155 staining throughout the anterior (Fig. 1C-E). All *fu* alleles also showed similar reductions in *ptc* expression indicated by a *ptc-lacZ* reporter gene at the AP border (Fig. 3.1 C'-E'). The increased Ci-155 staining extends far beyond the broadened stripe of *ptc-lacZ* in all cases, suggesting that increased spread of Hh is very unlikely to account for the observed increases in Ci-155 levels. Nevertheless, Ci-155 levels did appear to be highest within the extended range of Hh signaling in wing discs for all three mutant *fu* alleles. Contrary to expectations based on previously asserted differences between fu^{94} and fu^{RX15} (Wang and Holmgren, 1999; Lefers et al., 2001), we found no reproducible difference in the pattern of 2A1 elevation for three *fu* alleles encoding proteins of different length.

We also aimed to assay the level of Ci-155 with a different method by using a Ci transgene that carries a C-terminal Myc tag, which is lost when Ci-155 is processed to Ci-75 (Zhou and Kalderon, 2010). We used the *C765-GAL4* driver to express Myc-tagged Ci

transgenes throughout wing discs (Fig. 3.1F-I). In a wild-type background, UAS-Ci-Myc accumulated to the highest levels in posterior cells and at the AP border where Hh opposes Ci-155 processing (Fig. 3.1F). The level of Ci-Myc is lower in anterior cells because it is processed to a form lacking the Myc epitope (Smelkinson et al., 2007). In homozygous fu^{M1} mutant wing discs, Ci-Myc levels were greatly increased in anterior cells relative to wild-type wing discs, even far from the AP border (Fig. 3.1G). A variant of Cithat lacks two Cos2 binding domains (CiΔCDNΔCORD-Myc) is processed more efficiently than wild-type Ci-Myc (Zhou and Kalderon, 2010), providing a greater contrast in Myc levels between anterior cells and AP border cells of normal wing discs (Fig. 3.1H). In fu^{M1} mutant wing discs CiΔCDNΔCORD-Myc also showed increased Myc accumulation in all anterior cells relative to wild-type discs (Fig. 3.1I), indicating that Fu is required for efficient processing of both wild-type Ci and CiΔCDNΔCORD.

To test definitively whether the actions of Fused on Ci-155 processing were cell-autonomous we examined homozygous *fu* mutant clones in discs heterozygous for in fu^{M1}, fu^{RX2} and fu^{W3} alleles (Figs. 3.1A, J; 3.S1A-C). We found that the level of Ci-155 in anterior fu^{M1} clones was much higher than in the surrounding cells and similar to the level of Ci-155 at the AP border (Fig. 3.1J). We also saw strong elevation of Ci-155 in anterior fu^{M1} smo^{2} clones (Fig. 3.S1C), confirming that increased Ci-155 levels did not depend on any response to Hh. The level of Ci-155 was also clearly increased in anterior fu^{RX2} and fu^{W3} clones (Fig. 3.S1A, B). The level of Ci-155 in fu^{M1}, fu^{RX2} and fu^{W3} was similar to the level of Ci-155 at the AP border (internal control), suggesting that the alleles have equally strong effects on the processing of Ci-155. Thus, three *fu* alleles, encoding proteins of 80, 612 and 748 of the total 805 amino acids all showed a cell-autonomous defect in Ci-155

processing of a similar magnitude that does not depend on responding to Hh. All of these truncated Fu proteins lack a Cos2-binding domain (Robbins et al., 1997), suggesting that failure to bind Cos2 may be the key shared deficit.

The role of Fu in processing can be accessory to that of Cos2 such as supporting Cos2 stability (Liu et al., 2007), in which case supplying high amounts of Cos2 would compensate for the deficit in Fu. We thereforeexplored whether the apparent deficit in Ci-155 processing in fu^{M1} clones could be compensated by providing excess Cos2 (Fig. 3.1K). We used the *C765-GAL4* driver (at 29° C) to express a *UAS-Cos2* transgene evenly throughout the wing disc at levels much higher than endogenous Cos2. However, excess Cos2 did not reduce the Ci-155 elevation observed in anterior fu^{M1} clones. Thus, Fu has an important role in Ci-155 processing that is distinct from the role of Cos2.

Finally, we wished to determine whether Fu was essential for any Ci-155 processing by using a *hh-lacZ* repression assay, a direct and more sensitive measure of Ci-75 generation which uses the ability of Ci-75 to inhibit the expression of a *hh-lacZ* reporter (Methot and Basler, 1999). *Methot and Basler* previously did not detect *hh-lacZ* repression (Ci-75 generation) in posterior fu^{A} *smo* clones, which led them to conclude that Fu is essential for Ci-155 processing (Methot and Basler, 1999). We generated fu^{M1} *smo* mutant clones marked by the absence of Fu in animals that expressed wild-type Ci throughout the wing disc and carried a *hh-lacZ* reporter gene. We saw clear repression of *hh*-lacZ in the posterior fu^{M1} *smo* mutant clones consistent with generation of Ci-75 (Fig. 3.1L). The fu^{M1} allele theoretically encodes only a very short (80 aa) protein, and is therefore likely equivalent to a null allele. In combination with findings from 2A1 staining

experiments, this observation suggests that Ci-155 can be processed in the absence of Fu but processing is very inefficient.

Su(fu) controls the level of Gli3 protein, the principal source of Hh transcriptional repressors in vertebrates (Ingham and McMahon, 2001). Indeed, western blot analysis of mouse embryonic extracts from Su(fu)-null embryos showed a severe reduction in the level of Gli3 repressor (Wang, B. et al., 2000). The level of Ci-75 repressor was similarly reduced in the extracts from Su(fu)-null wing discs (Ohlmeyer and Kalderon, 1998). We therefore asked if removing this potential contribution of Su(fu) to Ci-155 processing would eliminate the residual processing that continues in the absence of Fu protein. To that purpose, we used Su(fu)-null wing discs expressing a wild-type Ci transgene to assay *hh-lacZ* repression in posterior fu^{M1} *smo* clones. We found that repression of *hh-lacZ* by Ci-75 persisted in fu^{M1} *smo* clones despite the removal of Su(fu) (Fig. 3.1M). Therefore, Cos2 is capable of some, albeit very inefficient, processing of Ci-155 in the absence of both Fu and Su(fu) proteins.

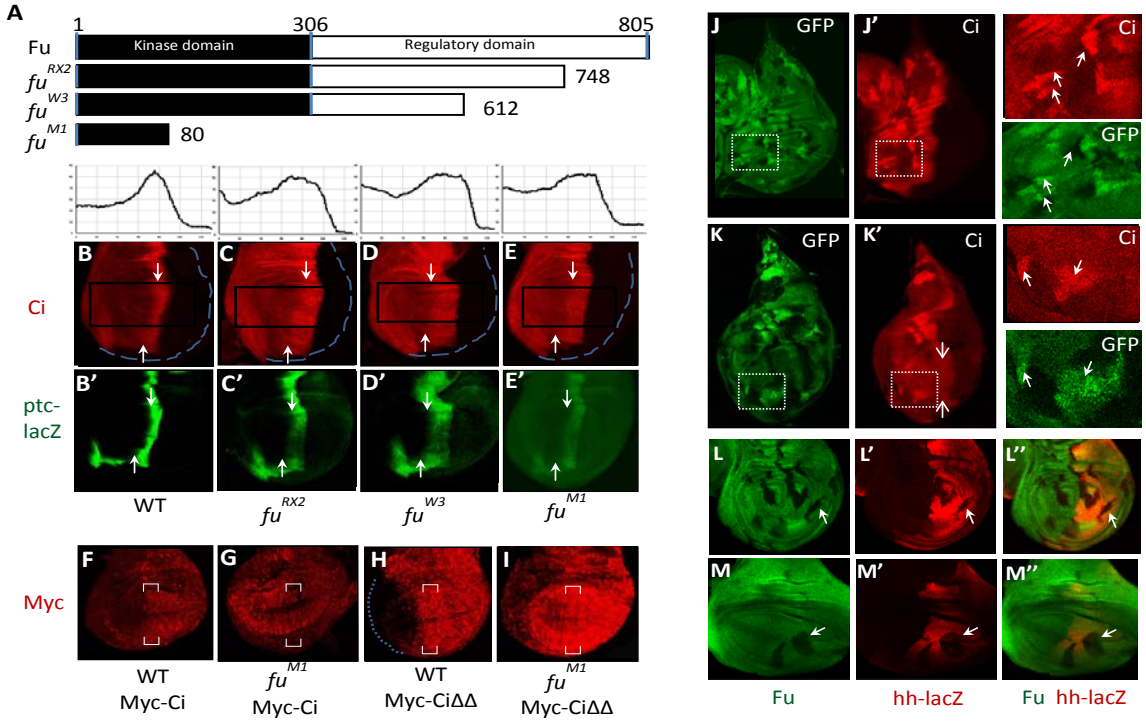


Figure 3.1. Efficient Ci-155 Processing in Drosophila Requires Fu C-terminus

(A) Diagram of Fu kinase and truncated proteins produced by the indicated alleles.

(B-E) Wing discs of *fu* alleles were stained with 2A1 antibody (red) to compare the profiles of Ci-155 accumulation. Areas enclosed by the rectangles were analyzed using *ImageJ* software to create quantitative intensity profiles of the fluorescent signal across the wing disc. Dashed outline marks the edge of the posterior compartment (right) that does not produce Ci-155. Fu alleles show ectopic accumulation of 2A1 in the anterior compartment (left). (B'-E') The region of Ci activation is marked by the expression of *ptc-lacZ* reporter and detected by an antibody to β-galactosidase (green), a protein product of *lacZ* gene. Arrows mark the anterior edge of *ptc-lacZ* expression. Fu alleles show a reduction in the level of *ptc-lacZ* expression. (F-I) Myc-tagged Ci transgenes were expressed in wing discs using *C765-GAL4* driver. Myc antibody (red) detects the full length form of Ci transgenes. Reduction in the level of Myc in the anterior cells (left) indicates efficient processing of Ci transgenes. Dotted outline in (H) indicated the anterior edge of the wing disc; white brackets mark the AP border. The level of Myc-Ci and Myc-CiΔΔ was low in the anterior region of the wing discs in Fu (WT) background and was elevated in the anterior cells in fu^{M1} background. (J, K) fu^{M1} clones were marked by two copies of GFP in a background that has one copy of GFP, and Ci-155 accumulation was

assayed in the most anterior clones (solid arrows) using 2A1 antibody (red). Insets with higher magnification are provided for the clones that are more difficult to discern. (J') Anterior fu^{M1} clones show strong accumulation of 2A1. (K') Cos2 transgene was expressed using *C765-GAL4* driver throughout a disc with fu^{M1} clones. Accumulation of 2A1 in anterior fu^{M1} clones was not rescued by Cos2 overexpression, which reduced the level of 2A1 at the AP border (open arrows). (L, M) *hh*-lacZ (red) is repressed by Ci repressor derived from UAS-Ci expressed using C765-GAL4 in posterior fu^{M1} *smo* clones (arrows), marked by loss of Fu staining (green) in otherwise normal discs (L, L", L") and homozygous $Su(fu)^{LP}$ (null) discs (M, M', M").

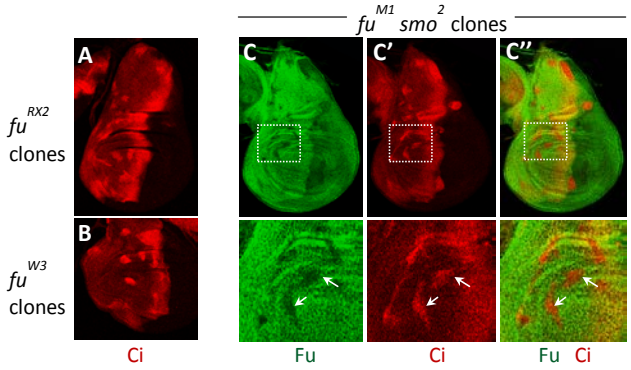


Figure 3.S1. Hh-Independent Cell-Autonomous Elevation of Ci-155 in *fu* Mutant Clones

(A, B) Wing discs with *fu* clones stained assayed for defects in Ci processing using 2A1 antibody (red). Strong accumulation of 2A1 in fu^{RX2} clones (A) and fu^{W3} clones (B) of anterior cells (right). (C, C', C'') Wing discs with fu^{M1} *smo* mutant clones that were negatively marked with Fu antibody (green) were assayed for Ci processing using 2A1 antibody (red). The level of 2A1 was elevated in anterior fu^{M1} *smo* mutant clones (C') that are magnified below with arrows indicating mutant clones.

Generating UAS-Cos2 with Impaired Binding to Fu

IP of wing disc extracts with Fu antibody followed by immunoblotting for Cos2 demonstrated that Fu associates robustly with Cos2 in wild-type wing discs but not in fu^{RX2} and fu^{W3} wing discs, implying that these alleles lack a critical Cos2-binding domain (Robbins et al., 1997). The deficits in Ci processing that we observed for these alleles and

for the more severely truncated fu^{M1} allele might be explained by the absence of this Cos2 binding domain. If so, then Cos2 which lacks the binding domain for Fu may show a similar deficit in Ci processing. Therefore, we decided to construct a Cos2 transgene that encodes a protein with impaired binding to Fu.

Ruel et al. co-transfected a series of Cos2 deletion constructs that carried a C-terminal Myc tag with an expression vector encoding wild-type Fu protein tagged with V5 epitope into S2 cells and precipitated the Cos2 protein variants with antibody against Myc (Ruel et al., 2007) (see p. 17 for diagram of Cos2). Immunoblotting with an antibody against V5 demonstrated that only the Cos2-Myc variants which contained a region of Cos2 between amino acids 543-605 were able to precipitate Fu-V5. Moreover, the fragment of Cos2-Myc that contained only this region (aa 543-605) precipitated with Fu-V5, suggesting that the peptide is sufficient for physical interaction between Cos2 and Fu (Ruel et al., 2007). To test whether amino acids 543-605 of Cos2 are also necessary for interaction with Fu, we made a construct that encoded a Cos2 protein lacking amino acids 543-605. Transfecting the construct into S2 cells did not produce the Cos2 protein variant in sufficient quantities for a binding assay, suggesting that amino acids 543-605 have a role in promoting Cos2 stability (data not shown). We therefore constructed transgenes that carried three smaller deletions in this region (540-560, 560-580, 580-600) for preliminary analysis. Although all three variants were stable in S2 cells (data not shown), only one of these deletions (540-560) resulted in a Cos2 variant with impaired binding to Fu (Cos2ΔFu). Our next step was to confirm that the deleted region (540-560) is necessary for Cos2 binding to Fu.

First, we assayed the ability of Flag-tagged Cos2ΔFu protein to pull down HA-Fu from S2 tissue cell extracts (Fig. 3.2A). Equal amounts of HA-tagged Fu DNA expression constructs were transfected into S2 cells alone, with Flag-Cos2 or with Flag-Cos2ΔFu transgenes. Immunoblotting with anti-HA antibody showed that the extracts of cells co-expressing Flag-Cos2 and HA-Fu contained significantly more HA-Fu protein than cell extracts with HA-Fu alone, or extracts with HA-Fu and Flag-Cos2ΔFu (Fig. 3.2A). Consistent with this observation, Cos2 has previously been reported to stabilize Fu in wing discs and tissue culture cells (Lum et al., 2003b; Ruel et al., 2003). We infer that stabilization of Fu by Cos2 in S2 cells requires Cos2 sequence from aa 540 to aa 560. The levels of Flag-Cos2ΔFu were also lower than those of Flag-Cos2 (Figs. 3.2A, 3.S2A), consistent with prior evidence that Cos2 can also be stabilized by binding to Fu in tissue culture cells (Liu et al., 2007).

Cell extracts were then immunoprecipitated with anti-Flag antibody and analyzed on a Western blot with anti-HA antibody. IP of Flag-Cos2 brought down far more HA-Fu than did Flag-CosΔFu when calculated as a percentage of total HA-Fu levels in the extract (7.8% for Flag-Cos2 and 1.1% for Flag-Cos2ΔFu). In a second experiment, when IP was conducted with equal but lower amounts of Cos2 transgenes, we detected almost no precipitation of HA-Fu by Flag-Cos2ΔFu (Fig. 3.S2A). Thus, deleting the Cos2 sequence from aa 540 to aa 560 severely reduced Cos2 binding to Fu in tissue culture cells expressing high levels of Cos2 and Fu proteins and eliminated apparent stabilization of Fu by Cos2.

We also investigated how the deletion of aa 540 to aa 560 affects the interaction between Cos2 and Fu in tissue culture cells by looking at co-localization between

Cos2ΔFu and HA-Fu in KC cells (Fig. 3.S2B-F). KC cells were transfected with GFP-Cos2 or GFP- Cos2ΔFu separately or together with Myc-Fu. Without GFP-tagged Cos2 transgenes, Myc-Fu distributed diffusely throughout the cell (Fig. 3.S2B). Transfection with GFP-Cos2 changed the distribution of Myc-Fu to punctate (Fig. 3.S2E), which is also the distribution of GFP-Cos2 and GFP-Cos2ΔFu in the absence of Myc-Fu (Fig. 3.S2C, D). There was significant positional overlap between Myc-Fu and GFP-Cos2 puncta (Fig. S2E). However, co-transfection with Cos2ΔFu had no effect on the normal distribution of Myc-Fu (Fig. 3.S2F). Thus, removing the Cos2 region between aa 540 and aa 560 impairs the association between Cos2 and Fu in S2 cells even when the two proteins are expressed at abnormally high levels.

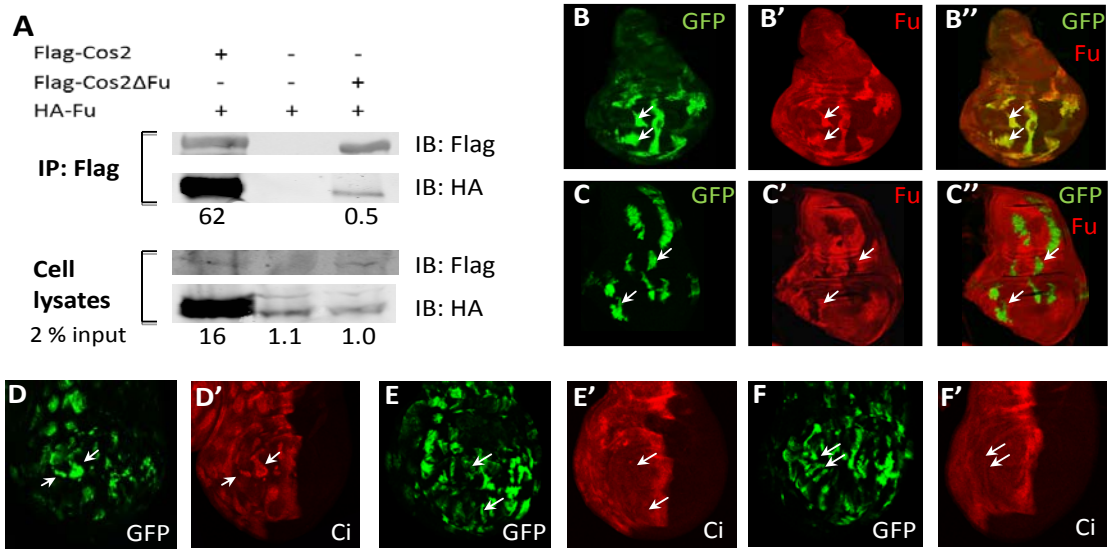


Figure 3.2. Fu Stabilization and Ci-155 Processing by Over-Expressed Cos2 Deficient for Fu Association

(A) S2 cells were transfected with HA-Fu (lanes 1 through 3) with Flag-Cos2 (lane 1) or Flag-Cos2ΔFu (lane 3). Cell lysates were immunoprecipitated (IP) with a mouse anti-Flag antibody, followed by immunoblotting (IB) with an anti-Flag or a rabbit anti-HA antibody. An aliquot of cell lysates, which corresponds to 2% or the material used in IP, was immunoblotted with an anti-Flag (top) or anti-HA (bottom) antibody. The band

intensities that are indicated under the bands were quantified with Lycor imaging software. Of note, the top band recognized by anti-HA antibody is non-specific. The lysates of cells that were transfected with Flag-Cos2 and HA-Fu contained significantly more HA-Fu than the lysates of cells with Flag- Cos2ΔFu or cell with HA-Fu alone (bottom). Flag-Cos2ΔFu was co-precipitated with 1.1%, and Flag-Cos2 with 7.8% of total HA-Fu in cell lysate. (B, C) The level of Fu was assayed using Fu antibody (red) in *cos2* clones (arrows) marked by GFP (green) expressing (B) UAS-Cos2 or (C) UAS-Cos2ΔFu. Fu level was high in *cos2* clones expressing wild type Cos2 (B', B'') and clearly reduced in clones with Cos2ΔFu (C', C''). (D-F) Ci-155 processing was assayed using 2A1 antibody (red) in *cos2* clones (arrows) that were marked by GFP (green), (D) alone or specifically expressing (E) Cos2ΔFu1, or (F) Cos2ΔFu2. High level of 2A1 in *cos2* mutant clones was reduced completely to the level of 2A1 in the surrounding tissue in the wing pouch by both transgenes (F, F).

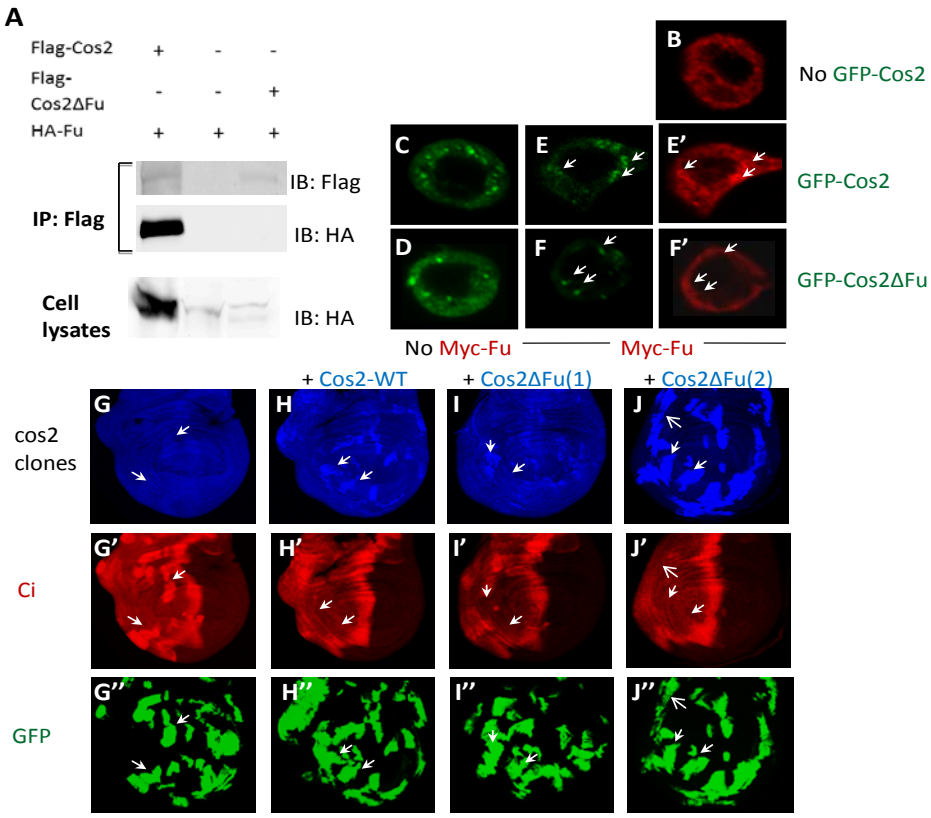


Figure 3.S2. Cos2ΔFu Does Not Change the Distribution of Fu in S2 cells, and the Level of Expression of Cos2ΔFu is Important for Ci Processing

(A) Another trial of the IP experiment from Fig. 3.2A. S2 cells were transfected with HA-Fu alone (lane 2) or together with either Flag-Cos2 (lane 1) or Flag-Cos2ΔFu (lane 3) in even but lower amounts (4 ng each compared to 8 ng in Fig. 3.2A). Low expression level of Flag-Cos2 variants prevented their detection by Flag antibody in the lysates, which contained 2% of the material used in IP. Flag antibody brought down similar amounts of Cos2 and Cos2ΔFu (IP:Flag, IB:Flag), but only the wild type Cos2 transgene associated with HA-Fu (IP:Flag, IB:HA). Cell lysates with Flag-Cos2 contained markedly more HA-Fu than those with Flag-Cos2ΔFu or HA-Fu alone (cell lysates, IB:HA). Top band recognized by anti-HA antibody is non-specific. (B-F) KC cells transfected with Myc-Fu (B), GFP-tagged Cos2 (C, E) or Cos2ΔFu (D, F) alone (C, D) or together with Myc-Fu (E, F) were immunostained with Myc antibody (red) to show the distribution of Fu. Myc-Fu formed puncta (arrows) in the presence of GFP-Cos2 (E'), but not in the presence of GFP-Cos2ΔFu (F'). Myc-Fu puncta colocalized with GFP-Cos2 puncta (E). (G-J) MARCM clones (solid arrows) without *cos2* activity (G) expressing the indicated Cos2 transgenes were assayed for the level of Cos2 expression using 7E11 antibody (blue) and for processing of Ci using 2A1 antibody (red). The level of Cos2-WT (H) was similar to the level of Cos2ΔFu1 (I) and lower than the level of Cos2ΔFu2 (J). High level of 2A1 in *cos2* mutant clones (G') was reduced completely to the level of 2A1 in the surrounding cells by all Cos2 transgenes, with more efficient rescue by Cos2ΔFu2 outside the wing pouch (open arrow).

Functional Characterization of UAS-Cos2ΔFu

Next, we looked at the functional significance of the Fu binding region of Cos2 in Drosophila. First we wanted to test if Cos2ΔFu can stabilize Fu in the wing discs. It has previously been shown that Fu levels are markedly reduced in *cos2* mutant clones (Lum et al., 2003b). We used the MARCM technique to make *cos2* clones marked by the presence of GFP (Lee and Luo, 2001). We then expressed either UAS-Cos2 or UAS-Cos2ΔFu in the *cos2* mutant clones and used an antibody to Fu to compare Fu protein levels (Fig. 3.2B, C). Expression of *UAS-Cos2* within *cos2* clones strongly increased the level of Fu in the clones compared to the level of Fu in the surrounding tissue (Fig. 3.2B). However, in *cos2*

clones expressing Cos2ΔFu, Fu levels were much lower than in surrounding tissue, as observed in *cos2* clones alone (Fig. 3.2C, Lum et al., 2003b). Thus, Cos2 binding to Fu through a region that includes aa 540 to aa 560 is essential to stabilize Fu protein in wing disc cells.

We then addressed the importance of the Cos2 region between aa 540 and aa 560 for Ci-155 processing. Cos2 is essential for Ci processing in flies, and *cos2* mutant clones show a strong elevation of Ci-155 (Wang, G. et al., 2000; Fig. 3.2D, Fig. 3.S2G'). We looked at the level of Ci-155 in anterior *cos2* mutant clones that specifically expressed either UAS-Cos2 or UAS-Cos2ΔFu. The wild type Cos2 transgene fully blocked the elevation of Ci-155 in anterior *cos2* clones, complementing the function of endogenous Cos2 (Fig. 3.S2H). Over-expressing Cos2 in *cos2* clones reduces the level of Ci-155 at the AP border (Zhou and Kalderon, 2010), and expressing excess Cos2 throughout the wing disc also reduced the level of Ci-155 at the AP border (Fig. 3.1K). These findings agree with prior inferences that excess Cos2 prevents normal activation of Smo and downstream responses to Hh, which include the inhibition of Ci-155 processing (Zhou and Kalderon, 2010).

Thus, to test whether Cos2ΔFu processing activity depended on the expression levels of Cos2ΔFu transgenes, we assayed complementation of *cos2* mutant clones using two transgenes with different expression levels (both in excess of physiological) (Fig. 3.S2I', J'). We found that both transgenes resulted in normal low levels of Ci-155 in anterior *cos2* mutant clones in the wing pouch (Figs. 3.2E, F). However, Ci-155 elevation in *cos2* clones outside the wing pouch was suppressed by one transgene (Cos2ΔFu2; Figs. 3.2F, 3.S2J') but not by the other (Cos2ΔFu1; Figs. 3.2E, 3.S2I'). We therefore compared

the level of expression of the two Cos2 transgenes (Fig. 3.S2G-J). Cos2 antibody was not sensitive enough to distinguish *cos2* mutant clones from the surrounding tissue with endogenous Cos2 (Fig. 3.S2G), but clearly detected excess Cos2 accumulation in *cos2* clones that expressed UAS-Cos2 (Fig. 3.S2H). The level of Cos2 due to expression of UAS-Cos2ΔFu1 in *cos2* clones (Fig. 3.S2I) was similar to the level produced by the wild type UAS-Cos2 transgene (Fig. 3.S2H), but much lower than the level of UAS-Cos2ΔFu2 (Fig. 3.S2J), consistent with the stronger processing activity of UAS-Cos2ΔFu2 (Figs. 3.2F, 3.S2J'). Thus, the expression level of Cos2ΔFu affects its ability to process Ci-155. Only levels much higher than normal supported efficient Ci-155 processing, judged by Ci-155 staining outside the wing pouch (Figs. 3.S2I, J). Moreover, the normal Ci-155 processing in the wing pouch supported by both UAS-Cos2ΔFu transgenes was in each case under conditions of excess Cos2 protein (Figs. 3.S2I', J'; 3.2 E, F).

Yeast two-hybrid analysis and GST-pull down assays have demonstrated that Fu and Ci can associate with Su(fu), a protein of unknown biochemical function (Monnier et al., 1998; Methot and Basler, 2000). We hypothesized that Su(fu) might provide a redundant route for recruiting Fu to Ci that would be revealed if Fu could not be recruited by Cos2. We therefore assayed Ci-155 processing by Cos2ΔFu in the absence of Su(fu).

We used the *hh-lacZ* reporter to test for the generation of Ci-75 repressor by Cos2ΔFu in posterior *smo cos2* clones that specifically express Ci in the presence of absence of Su(fu) (Fig. 3). Even in the absence of Su(fu), UAS-Cos2ΔFu2 supported Ci-75 production sufficient to block *hh-lacZ* expression in *smo cos2* mutant clones (Fig. 3.3C). The same result was observed when we expressed a derivative of Ci lacking the CORD Cos2-binding domain (Zhou and Kalderon, 2010) in place of wild-type Ci in the *hh-lacZ*

repression assay (Fig. 3.3D). Thus, Ci can still be processed to some degree by over-expressed Cos2 that is highly deficient for Fu binding in the absence of Su(fu) and in the absence of one of the two major domains in Ci that associate with Cos2.

Expressing Cos2ΔFu in vast excess of physiological levels supported processing of Ci-155 even in the absence of Su(fu) (Fig. 3.3), whereas reduced Ci-155 processing was observed outside the wing pouch region for Cos2ΔFu transgene with lower, but still greater than physiological expression levels (Figs. 3.2E, 3.S2I). We therefore wished to test the activity of a Cos2ΔFu transgene expressed at precisely physiological level, which has not been possible with UAS-Cos2 transgenes.

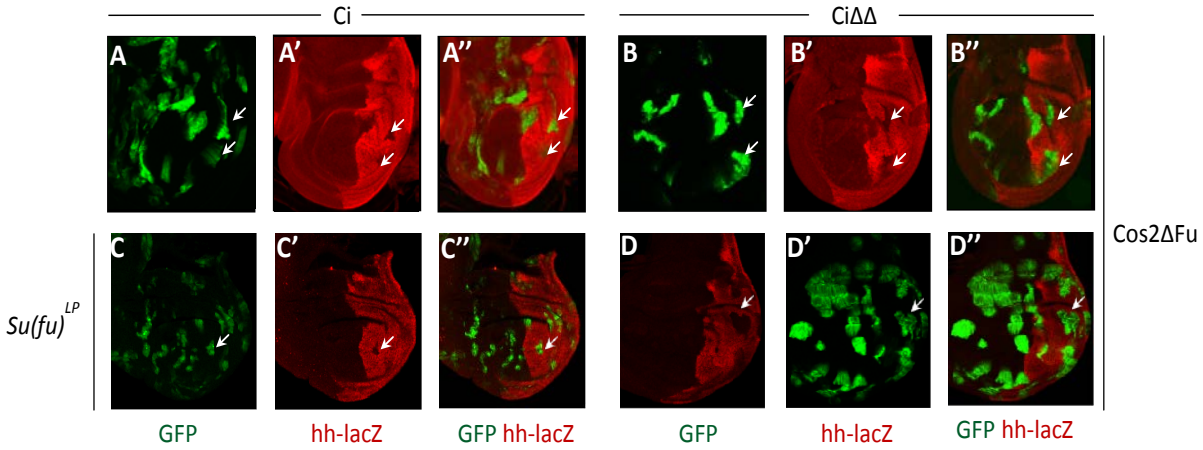


Figure 3.3. Ci-155 Processing by Cos2 Deficient for Fu Binding Does Not Require Su(fu)

(A-D) Repression of *hh-lacZ* (red), indicating Ci processing, was seen in *smo cos2* mutant clones expressing UAS-CosΔFu(2), marked by GFP (green) for both (A, C) wild-type Ci and (B, D) CiΔΔ in (A, B) otherwise normal discs and (C, D) $Su(fu)^{LP}$ discs.

Do Physiological Levels of Cos2ΔFu Support Processing of Ci-155?

We chose to construct a genomic Cos2ΔFu transgene that includes normal Cos2 regulatory sequences in order to test the importance of binding to Fu at physiological Cos2 expression levels. Analysis of molecular lesions shared by amorphic *cos2* alleles and deletions that result in the loss of Cos2 function identified a 6.5-kb genomic fragment (K6.5) which rescued the viability of *cos2* embryos (Sisson et al., 1997). More careful analysis of pK6.5 transgene properties showed that the fragment also rescued larval lethality and suppressed wing duplications in *cos2* loss-of-function and null trans-heterozygotes (Ho et al., 2005). We therefore based our genomic Cos2 construct on the K6.5 fragment that consists of about 0.8 kb upstream of the transcriptional start site and 1.7 kb downstream of the transcribed sequences present in cDNAs (Fig. 2.1).

Analysis of the Cos2 sequence revealed a coiled-coil domain which mediates formation of Cos2 homodimers, and similarity to the kinesin superfamily, many members of which are constitutively dimeric (Sisson et al., 1997). IP of Cos2 molecules that carried two different tags from extracts of Kc cells showed robust co-precipitation, indicating constitutive dimers or higher oligomers (Zhou and Kalderon, 2011), whereas similar experiments performed by a different group in S2R+ cells showed that Cos2 is capable of dimerization, but only under conditions of Smo activation (Ranieri et al., 2012). Therefore, transgenic Cos2 variants might have dominant negative activity by forming dimers with endogenous Cos2. In an effort to avoid possible dominant negative effects, we introduced an excisable transcriptional terminator into the first intron of our genomic Cos2 transgenes, as described below (see also Fig. 2.1).

We constructed conditional genomic Cos2 transgenes by introducing three modifications into the original K6.5 fragment. First, we mutated the 5' KpnI site to SphI to allow cloning into the pUAS-attB vector, which permits integration of the transgene at a specific chromosome site. Second, we introduced an AvrII restriction site into the first intron of K6.5. Lastly, we cloned the 3-kb transcription terminator (FC22, provided by G. Struhl (Struhl and Basler, 1993)) with terminal NheI sites into the AvrII entry site on pUAS-attB-K6.5, producing g<term<Cos2. Cloning of the transcriptional terminator damaged the 3' end of the terminator cassette on the initial attempts. We therefore tried to circumvent this step by using recombineering, a less conventional cloning method based on phage λ recombination system (Copeland et al., 2001). However, conventional cloning was ultimately successful (see also *Recombineering* in the Materials section).

Once we established Drosophila stocks with g<term<Cos2 transgene insertions at the attB site at position *86Fb* on the third chromosome we tested whether expression of the Cos2 transgenes did produce any dominant phenotypes. We induced the excision of the transcriptional terminator cassette in females carrying one copy of $cos2^2$ allele (amorph) and a *hs-flp* transgene by delivering a heat shock during the late 3rd instar. We expected that a high proportion of future germline stem cells would excise the transcription termination cassette through recombination at the flanking FRT sites, leaving a gCos2 transgene with only FRT and short additional sequence in the first intron. Female progeny were collected and crossed to males which were also heterozygous for the $cos2^2$ allele. We then looked for progeny which inherited both copies of $cos2^2$ allele due to viability rescue by the genomic Cos2 fragment without the transcriptional terminator. Wild type genomic Cos2 (gCos2-WT) fully rescued animal viability, but gCos2ΔFu did not rescue $cos2^2$-

mutant larvae to adulthood. However, genotyping of individual progeny heterozygous for *cos2* revealed that most had excised the transcriptional terminator cassette. The resulting gCos2ΔFu transgene without the terminator could be propagated without any obvious dominant negative effect on viability, fertility or wing patterning (see also *Germline Excision of Transcriptional Terminator and Genotyping* in the Materials section).

We aimed to test the effect of disrupting Cos2-Fu interaction on Ci processing in a more physiological setting (by using genomic Cos2 transgenes). To that purpose, we examined third instar wing discs from animals homozygous for *cos2* or containing *cos2* homozygous clones and also carrying either the active gCos2-WT or gCos2ΔFu transgenes (after excision of the transcriptional terminator) (Fig. 3.4). As expected from full rescue of adult viability and patterning, discs entirely mutant for *cos2* were fully rescued by gCos2-WT with elevated Ci-155 and *ptc-lacZ* staining confined to the AP border (Fig. 3.4A, A'). However, *cos2* mutant discs with gCosΔFu had very high Ci-155 levels throughout the anterior compartment and were distorted, with expanded anterior compartments (Fig. 3.4B). That phenotype is consistent with very inefficient or absent Ci-155 processing and the consequent induction of *dpp* for lack of Ci-75 repressor (Méthot and Basler, 2001). Similarly, *cos2* mutant clones in discs with gCos2-WT had normal low levels of Ci-155 (Fig. 3.4D), whereas *cos2* clones in discs with gCosΔFu had very high Ci-155 levels (Fig. 3.4E), and frequently produced clearly expanded anterior compartments. Those clones did not, however, express significant levels of *ptc-lacZ* (Fig. 3.4H), showing that the increased Ci-155 was not as active as in *cos2* clones with no Cos2 transgene, in which *ptc-lacZ* is strongly induced (Wang and Holmgren, 2000). Thus, CosΔFu expressed at physiological levels appears to be extremely defective in supporting Ci-155 processing (Fig. 3.4B, E'),

implying that the more efficient Ci-155 processing seen for UAS-Cos2ΔFu transgenes owed much to expression at higher than normal levels. To see if gCosΔFu could support any Ci-155 processing we examined posterior *smo cos2* clones in animals that expressed CiΔCDNΔCORD throughout the wing disc (Fig. 3.S4A). We observed clear *hh-lacZ* repression in the wing pouch (Fig. 3.S4A'), showing that physiological levels of Cos2ΔFu can support some Ci-155 processing, albeit much less efficiently than normal.

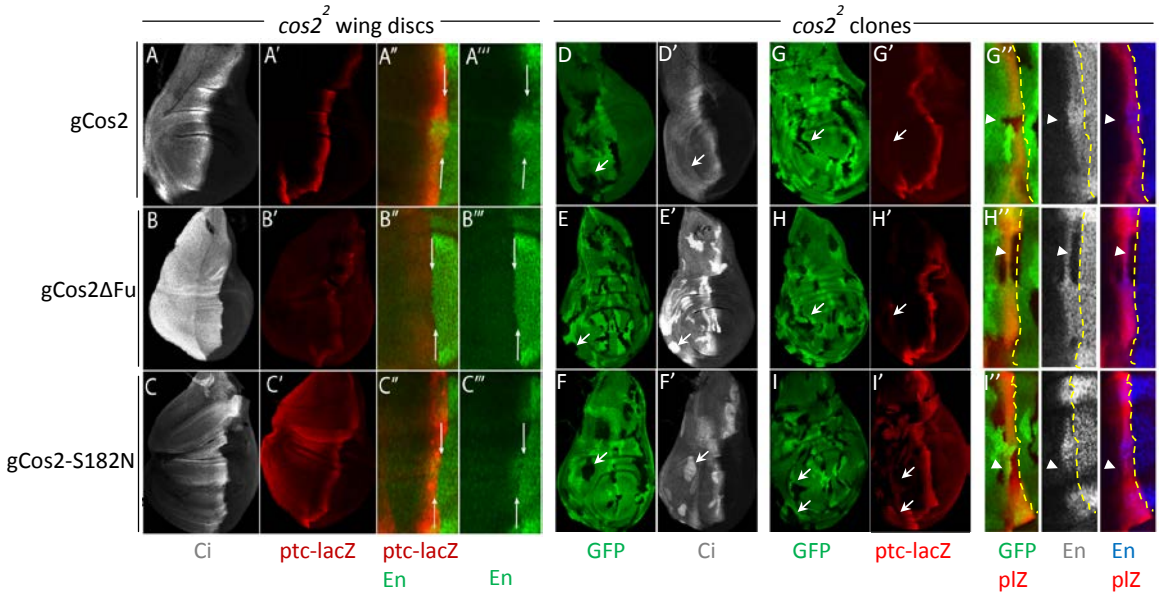


Figure 3.4. Properties of Cos2 Variants Deficient for Fu or Ci CORD Binding When Expressed at Physiological Levels

(A-C) Wing discs homozygous for *cos2*^{2} with one copy of genomic transgenes for (A) wild-type Cos2, (B) Cos2ΔFu or (C) Cos2-S182N, showing (A-C) Ci-155 staining (white), (A'-C') *ptc*-lacZ staining (red) and (A"-C") En staining (green) alone [A'''-C'''] or together [A''-C''] with *ptc*-lacZ (red) to reveal the exact position of the AP border (arrows) as the posterior (right) edge of *ptc*-lacZ staining. (D-I) Wing discs with *cos2*^{2} mutant clones (arrows), marked by loss of GFP (green) and one copy of a genomic transgene for (D, G) wild-type Cos2, (E, H) Cos2ΔFu or (F, I) Cos2-S182N, showing (D'-F') Ci-155 staining (white), (G'-I') *ptc*-lacZ staining (red). (I, I') gCos2-S182N does not prevent the induction of ectopic *ptc*-lacZ in anterior *cos2*^{2} clones. (G"-I") Close-ups of the AP border in the wing pouch are shown for [middle] En staining alone

(white) or [right] together (blue) with *ptc*-lacZ (red) to mark the AP border (dashed lines) and [left] *ptc*-lacZ with GFP to mark the location of AP border GFP-negative clones (arrowheads).

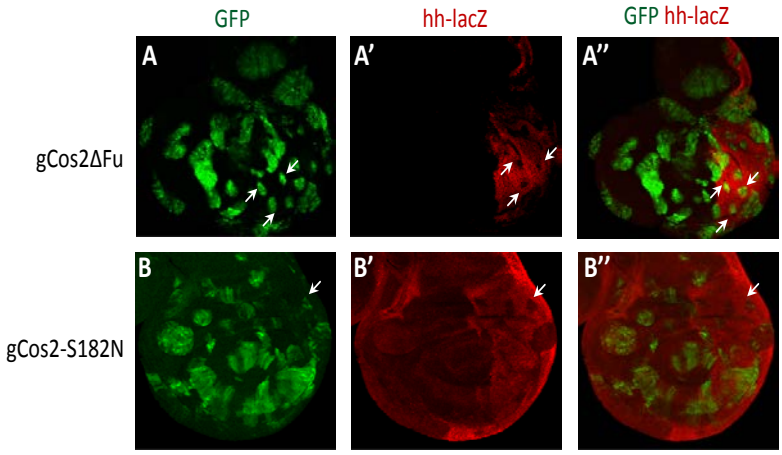


Figure 3.S4. Ci-155 Processing by Cos2 Variants Deficient for Fu or Ci CORD Binding when Expressed at Physiological Levels

(A, B) Repression of *hh*-lacZ (red), indicating Ci-155 processing, was seen in *smo cos2* mutant clones, marked by GFP (green) for wing discs expressing UAS-CiΔΔ and carrying genomic transgenes for both (A) Cos2ΔFu and (B) Cos2-S182N.

Roles of other Cos2 Variants in Supporting Ci-155 Processing

The ability of a small group of Cos2 variants to support Ci-155 processing has previously been assayed using UAS-Cos2 transgenes (Lum et al., 2003b; Ho et al., 2005; Zhang et al., 2005; Ruel, et al., 2007). As can be seen with CosΔFu, it is critical to re-evaluate these findings using Cos2 variants expressed at physiological levels. We therefore constructed additional gCos2 transgenes, inserted at the same cytological location, with mutations encoding four specific alterations - S182N, S572A, S572D, S572A+S931A, and S931A. In all cases we were able to excise the transcriptional

terminator cassette and propagate the resulting gCos2 transgenes with no adverse effects indicating any dominant phenotypes.

The S182N mutation in the Cos2 motor domain is thought to impair the ability of Cos2 to bind or hydrolyze ATP and was found to block the ability of Cos2 to move along microtubules in tissue culture cells (Ho et al., 2005; Farzan et al., 2008). GST-Ci binding assays demonstrated that Cos2-S182N also fails to interact with the C-terminal Cos2 binding domain on Ci (CORD) (Zhou and Kalderon, 2011). In those CORD domain binding assays wild-type Cos2 binding is greatly stimulated by ATP, suggesting that Cos2 S182N does not adopt a suitable conformation for binding CORD that is normally dependent on ATP binding. High level expression of Cos2 S182N from a UAS-driven transgene was previously shown to support very limited processing of wild-type Ci-155 but considerably more efficient processing of CiΔCDNΔCORD or CiΔCORD, suggesting that CORD domain restricts Ci-155 processing, and that Cos2 binding to CORD relieves that restriction (Zhou and Kalderon, 2010). Genomic Cos-S182N (gCos2-S182N) was able to support processing of CiΔCDNΔCORD sufficient for strong *hh-lacZ* repression in posterior *smo cos2* clones (Fig. 3.3S4B), confirming previous results with more highly expressed UAS-Cos2S182N (Zhou and Kalderon, 2010). In addition, gCos2-S182N failed to reduce the ectopic accumulation of Ci-155 in anterior *cos2* clones (Fig. 3.4F), exactly as observed for gCosΔFu (Fig. 3.4E). However, in contrast to observations with gCosΔFu, there was clear ectopic *ptc-lacZ* expression in *cos2* clones in the presence of gCos2-S182N (Fig. 3.4H, I), albeit lower than in *cos2* clones with no Cos2 rescue transgene (Wang and Holmgren, 2000). Thus, neither gCosΔFu nor gCosS182N support efficient Ci-155 processing when expressed at physiological levels but Cos2 S182N is additionally

defective in silencing the increased levels of Ci-155. This suggests that binding of Cos2 to the CORD domain of Ci not only promotes Ci-155 processing but may also contribute towards silencing Ci-155, perhaps by retaining more Ci-155 in the cytoplasm (Wang, G. et al., 2000).

Two Fu-dependent phosphorylation sites, S572 and S931, have been mapped on Cos2 and investigated with regard to their role in responding to activated Fu (Ranieri et al., 2012). Key variants used for those investigations have S572A, S572D and S931A substitutions. Although the primary objective is to test the response of these variants to Hh (see next section), it is important to determine their baseline processing function in anterior cells distant from Hh. Based on over-expressed cDNAs it was previously suggested that Cos2-S572A was somewhat deficient for Ci-155 processing and Cos2-S572D was significantly poorer at supporting Ci-155 processing (Ruel et al., 2007). Those properties were suggested to correlate with poorer association of Cos2 S572D than Cos2 S572A or wild-type Cos2 with Ci-155, as determined by co-precipitation from *cl8* cells (Ruel et al., 2007). We found that gCos2 S572A, S572D and S931A all supported efficient Ci-155 processing as judged by 2A1 staining of Ci-155 in *cos2* mutant clones (Fig. 3.S6). Those clones had the same Ci-155 staining as surrounding tissue and no sign of ectopic *ptc-lacZ* or any indication of altered wing disc growth, indicative of ectopic *dpp* (Fig. 3.6A'-D'). Hence, these Cos2 variants appear to be more active in anterior cells than previously claimed and all process and silence Ci-155 efficiently, providing an excellent background for testing whether their activities are regulated in response to Hh.

Roles of Cos2 in Responding to Hh

Loss of expression of anterior *engrailed* (*en*), a transcriptional target induced only by high-level Hh signaling, in *cos2* clones at the AP border was the first indication for the involvement of Cos2 in responding to Hh (Wang, G. et al., 2000). Expressing an activated form of Smo, SmoD123 (Jia et al., 2004), in *smo cos2* clones did not rescue the expression of anterior *en*, suggesting that activated Smo requires Cos2 to activate Hh transcription targets (Zhou and Kalderon, 2011). This role of Cos2 in responding to Hh includes the activation of Fu by promoting cross-phosphorylation of Fu molecules (Zhou and Kalderon, 2011; Shi et al., 2011). Indeed, increasing the level of Cos2 in S2 cells also increased the FRET between CFP-Fu and YFP-Fu, and Fu-dependent phosphorylation at S159 is essential for rescue of AP border *ptc-lacZ* and En expression in fu^{mH63} wing discs (Zhou and Kalderon, 2011; Shi et al., 2011). Hh stimulation increased the stability of Smo-Cos2 complexes in Cl8 cells, and these complexes were shown to accumulate at the membranes of Hh-receiving cells in Drosophila embryos, suggesting that Cos2 promotes Smo accumulation at the plasma membrane (Ruel et al., 2003). It was also shown that, if the amount of transfected Smo is fixed, increasing the levels of co-transfected Cos2 initially causes a rise in *ptc*-luciferase reporter activity in Cl8 cells, suggesting that Cos2 is required for activation of Smo (Lum et al., 2003b). Therefore, Cos2 may play a role in Smo activation, possibly by promoting Smo accumulation at the plasma membrane (Ruel et al., 2003; Lum et al., 2003b). It has previously not been possible to investigate the role of Cos2 variants in responding to Hh at the AP border of wing discs because UAS-Cos2 transgenes were invariably expressed at higher levels than normal and dominantly

inhibited Hh signaling (Ruel et al., 2007; Raisin et al., 2010). Our genomic Cos2 transgenes now present an opportunity to investigate how Cos2 responds to Hh.

First, we aimed to establish that wild type genomic Cos2 complements the roles of endogenous Cos2 in supporting Hh transcriptional response. We therefore showed that one copy of the wild-type gCos2 transgene fully substituted for *cos2* mutation at the AP border (Fig. 3.4A). Expression of gCos2 throughout the wing disc rescued the expression of anterior *en* at the AP border in both *cos2* clones and in animals homozygous for *cos2* (Fig. 3.4A, G). By contrast, gCos2ΔFu did not support En expression at the AP border in *cos2* mutant clones (Fig. 3.4H) or discs entirely mutant for Cos2 (Fig. 3.4B'', B'''). Moreover, in such *cos2* mutant cells *ptc-lacZ* was only expressed at a low level, indicating a severe reduction in Ci-155 activation (Fig. 3.4H'). Since *cos2* mutant cells with gCos2ΔFu in anterior cells away from the AP border expressed no detectable *ptc-lacZ* (Fig. 3.4H), there was clearly some residual response to Hh supported by CosΔFu. In both *cos2* mutant clones and homozygous *cos2* mutant whole discs in the presence of gCosΔFu, Ci-155 levels were equally high in anterior and AP border cells and much higher than normally seen in AP border cells (Fig. 3.4B, E'), possibly because strong Hh signaling, supported by Fu activation, induces turnover of Ci-155 by a Cul3-dependent mechanism that is independent of Ci-155 processing (Zhang et al., 2006). Altogether, the phenotype of AP border cells expressing only gCos2ΔFu appears identical to that of cells lacking Fu kinase activity (Ohlmeyer and Kalderon, 1998): abnormally high Ci-155, greatly reduced *ptc-lacZ* and no En induction.

Next, we aimed to identify the origin of the defect in Fu activation contributed by Cos2ΔFu. One possibility is that Cos2ΔFu prevents normal activation of Smo and

subsequent Smo accumulation (Denef et al., 2000). To address this hypothesis, we compared the level of Smo protein in *cos2* homozygous discs expressing either wild type gCos2 or gCos2ΔFu (Fig. 3.S5). For both conditions, we saw a normal Smo accumulation in the regions of high Hh signaling (posterior compartment and edge of the AP border), suggesting that gCos2ΔFu supports this component of Smo activation (Fig. 3.S5A, B). Next, we tested the response of gCos2ΔFu in *smo cos2* clones to activated Smo (SmoD1-3, (Jia et al., 2004)), which causes strong ectopic induction of *ptc-lacZ* in the presence of wild type gCos2 (Fig. 3.5A). Genomic Cos2ΔFu blocked the induction of *ptc-lacZ* in response to SmoD1-3, suggesting that this variant is defective in responding to activated Smo (Fig. 3.5B). Therefore, although gCos2ΔFu supports the accumulation component of Smo activation that occurs based on some induction of *ptc-lacZ* at the AP border (Fig. 3.4H'), gCos2ΔFu is defective in transducing Smo activation. We tested if supplying excess levels of Fu, a key target of activated Smo (Zhou and Kalderon, 2011), could complement the activation defect of gCos2ΔFu (Fig. 3.5D-F). We expressed an active form of Fu that does not require Smo (GAP-Fu, (Claret et al., 2007)) in *smo cos2* clones and found that strong *ptc-lacZ* was induced in the clones when the wing discs contained gCos2ΔFu or wild type gCos2 (Fig. 3.5D, E).

Considerable evidence has previously been presented that Fu activation requires cross-phosphorylation between Fu molecules that is promoted by association of inactive Fu molecules with Cos2 (Shi et al., 2011; Zhang et al., 2011; Zhou and Kalderon, 2011). The properties of gCos2ΔFu at the AP border provide strong support for the critical role of Cos2 binding to Fu in allowing Fu activation.

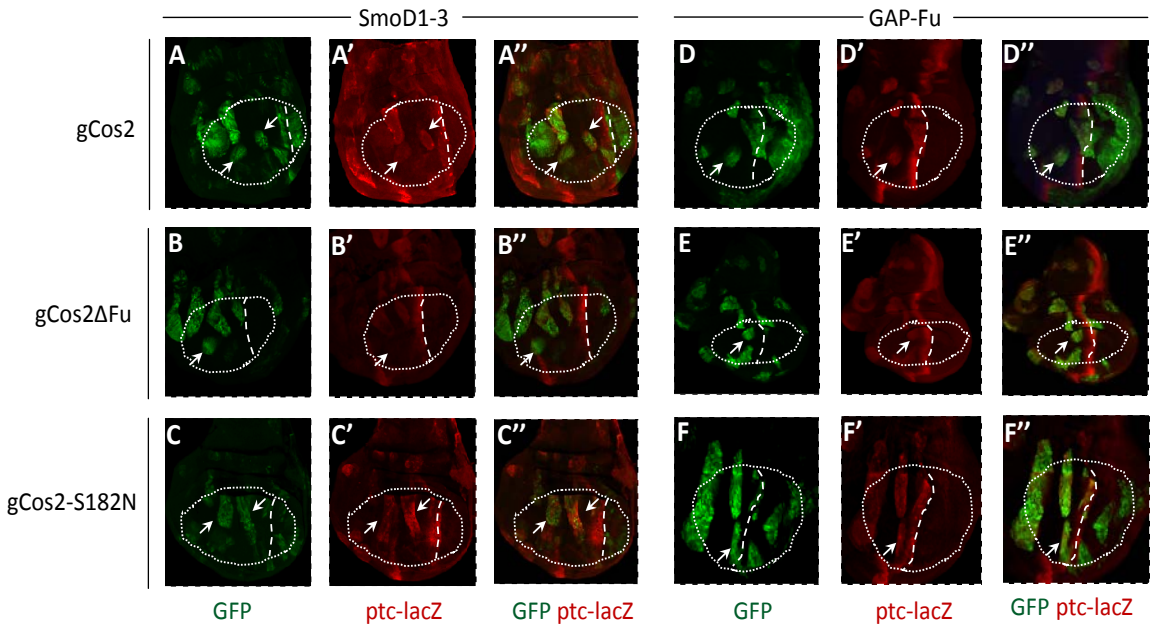


Figure 3.5. Cos2 with Impaired Fu Binding Fails to Activate Fu in Response to Activated Smo

(A-F) Ectopic *ptc-lacZ* (red) induction in anterior *smo cos2* clones (arrows), marked by GFP (green), by (A-C) UAS-SmoD1-3 or (D-F) UAS-GAP-Fu in discs carrying genomic transgenes for (A, D) wild-type Cos2, (B, E) Cos2ΔFu or (C, F) Cos2-S182N. Circular dotted lines indicate the wing pouch and dashed vertical lines indicate the AP border, separating the anterior compartment (to the left); arrows indicate anterior clones that originated in the wing pouch. Induction of *ptc-lacZ* by SmoD1-3 is impaired for (B) Cos2ΔFu compared to (A) Cos2-WT or (C) Cos2-S182N, while (D-F) *ptc-lacZ* induction by GAP-Fu is similar for all three Cos2 transgenes.

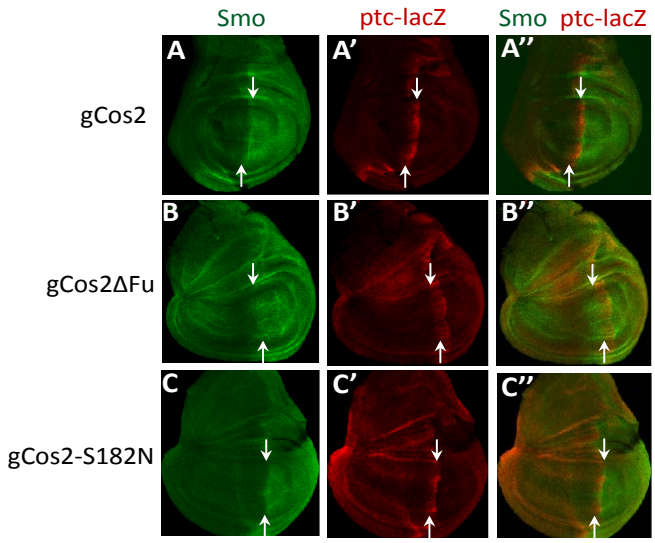


Figure 3.S5. Smo Stabilization by Cos2 Variants Deficient for Fu or Ci CORD Binding

(A-C) Smo staining (green) in *cos2* mutant discs is highest in the posterior compartment and the posterior edge of the AP border, as normal, for discs carrying genomic transgenes for (A) wild-type Cos2 and (C) Cos2-S182N and (B) Cos2ΔFu. (A'-C') Staining with *ptc-lacZ* (red) marks the AP border (arrows).

The role of Fu-dependent Cos2 Phosphorylation Sites in Responding to Hh

The Therond group developed phospho-specific antibodies against Cos2 residues Ser572 and Ser931 and confirmed that these phosphorylation events depend on the activity of Fu kinase (Ruel et al., 2007; Ranieri et al., 2012). They also used these antibodies for immune fluorescence analysis in the wing discs to compare the distribution of phosphorylated Cos2 for the two phosphorylation sites. Ser572 phosphorylation was associated with the entire region of active Hh signaling including the region of Ci-155 stabilization, while Ser931 phosphorylation did not expand into the anterior beyond the narrow stripe of cells associated with the highest level of Hh (Ruel et al., 2007). IP experiments and western blot analyses of protein levels showed that Ser572 phosphorylation reduces the association between Cos2 and Ci with accompanying

destabilization of Cos2-Fu complex, suggesting that the event opposes the nucleation of Ci processing complex and supports Cos2 degradation, amplifying the activation of Smo (Ruel et al., 2003). The assumption that Ser572 phosphorylation opposes processing and leads to Ci stabilization was later supported when preventing phosphorylation of this residue by Alanine substitution blocked the accumulation of Ci-155 in *cos2* clones in response to activated Fu (FuEE) (Zhou and Kalderon, 2011). However, this contribution of Fu kinase to Cos2-mediated processing is not essential for Hh-dependent Ci stabilization, as the level of Ci-155 at the AP border of fu^{mH63} wing discs without active Fu kinase is still very high (Ohlmeyer and Kalderon, 1998). In-vitro activity assays using *ptc*-luciferase reporter have demonstrated that simultaneous substitution of both Ser572 and Ser931 for Alanine produced a Cos2 variant which was unable to support transcriptional response to Hh, suggesting that the role of Fu-mediated Cos2 phosphorylation in responding to Hh could involve stimulating Ci activity rather than stabilizing Ci-155 (Ranieri et al., 2012).

We were hoping to replicate this finding in a more physiological setting by substituting endogenous Cos2 for genomic Cos2 transgenes in *cos2* clones at the AP border. To our surprise, phospho-deficient variants of gCos2 that were sequenced to verify the mutations were able to support normal transcriptional response to Hh, and did not show any defects in processing of Ci-155 (Figs. 3.6, 3.S6). Specifically, these transgenes (gCos2-S572A, gCos2-572D, gCos2-S931A and gCos2-572A 931A) supported normal expression of *ptc-lacZ* and anterior En in *cos2* clones at the AP border (Fig. 3.6A-D), showed normal Ci-155 processing in anterior *cos2* clones (Fig. 3.S6) and rescued *cos2* animals to adulthood. Thus, S572 and S931 play no essential, non-redundant role in Hh signaling. In finding no essential role for Ser572 and Ser931 residues of Cos2 for Ci

activation, we made progress towards answering whether Cos2 phosphorylation by Fu is a cause or an outcome of Smo-Cos2-Fu complex stabilization (Chen and Jiang, 2013). Wild type behavior of Cos2 phosphorylation mutants is strong evidence for the latter scenario. It is still possible that Cos2 phosphorylation confers a minor benefit to stability of Smo-Cos2-Fu complex by reinforcing the binding of Cos2 to Fu, and thereby the stability of Fu, hypothesis supported by Cos2 binding analyses in tissue culture cells using phospho-specific antibodies (Ruel et al., 2007; Liu et al., 2007). It is also known that Fu kinase activity has a role in Hh-dependent Ci stabilization which involves phosphorylation of Ser572 on Cos2 (Zhou and Kalderon, 2011).

We decided to test this finding under more physiological settings by measuring the response to activated Fu (GAP-Fu, Claret et al., 2007) using genomic Cos2 variants (Fig. 3.6I-K). Expressing normal gCos2 together with GAP-Fu in anterior *smo cos2* clones resulted in a slight increase in the level of Ci-155 and strong induction of *ptc-lacZ* (Fig. 3.6L), consistent with previous observations that GAP-Fu does not require Smo to stabilize Ci-155 and to induce *ptc* expression (Claret et al., 2007). The transcription response to GAP-Fu in anterior *smo cos2* clones was not prevented by co-expressing gCos2-S572A or gCos2-S931A (Fig. 3.6J, L), but the slight increase in Ci-155 was absent for gCos2-S572A (Fig. 3.6I). Consistent with this observation, Cos2 transgene with both substitutions also prevented Ci-155 stabilization in anterior *smo cos2* clones expressing GAP-Fu (Fig. 3.6K), although strong induction of *ptc-lacZ* was observed in all cases (Fig. 3.6E-H). Thus, although neither residue is necessary for Ci activation in response to activated Fu, Ser572 has a role in Fu-dependent Ci stabilization. This finding is consistent with previous

experiments that used another form of activated Fu, FuEE (Zhou and Kalderon, 2011), which we are hoping to test in the future.

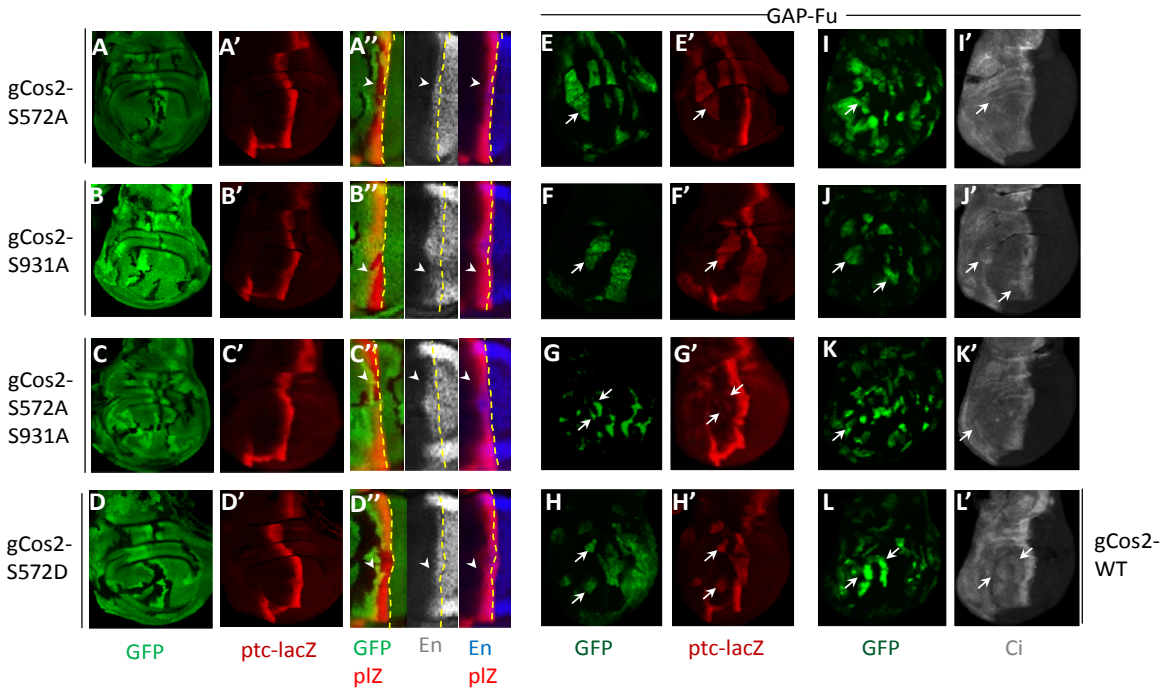


Figure 3.6. Cos2 Phosphorylation Site Variants Support Normal Hh Signaling and Ci-155 Stabilization in Response to Fu-Dependent Cos2 Phosphorylation of Ser572

(A-D) Wing discs with *cos2* mutant clones, marked by loss of GFP (green) and one copy of a genomic transgene for (A) Cos2-S572A, (B) Cos2-S931A, (C) Cos2-S572AS931A or (D) Cos2-S572D, showing (A'-D') no changes in *ptc*-lacZ staining (red) and (A''-D'') Close-ups of the AP border in the wing pouch are shown for [middle] En staining alone (white) or [right] together (blue) with *ptc*-lacZ (red) to mark the AP border (dashed lines) and [left] *ptc*-lacZ with GFP to mark the location of AP border GFP-negative clones (arrowheads). (E-L) Anterior *cos2* mutant clones expressing UAS-GAP-Fu (arrows), marked by GFP (green) and one copy of a genomic transgene from (A-C) or genomic Cos2-WT (H, L), show (E'-H') ectopic *ptc*-lacZ (red) induction, and (I'-L') Ci-155 (white) stabilization contingent on the presence of Ser572.

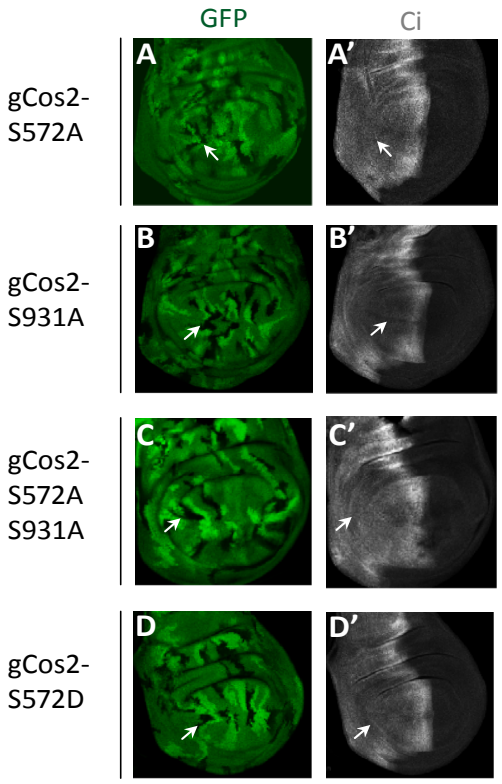


Figure 3.S6. Cos2 Phosphorylation Site Variants Support Normal Ci-155 Processing

(A-D) Wing discs with *cos2* mutant clones (arrows), marked by loss of GFP (green) and one copy of a genomic transgene for (A) Cos2-S572A, (B) Cos2-S931A, (C) Cos2-S572AS931A or (D) Cos2-S572D, showing (A'-D') no significant changes in Ci-155 staining (white).

Chapter IV

Discussion

Atypical kinesin Cos2 is essential for both processing and activation of Ci-155 (Sisson et al., 1997; Wang, G. et al., 2000). The role of Cos2 in Ci-155 processing involves scaffolding processing-promoting kinases (Zhang et al., 2005) and binding to Ci-155 through CORD and Znf regions (Wang, G. et al., 2000; Zhou and Kalderon, 2010). Cos2 acts in a complex with Fu kinase (Liu et al., 2007), and their contributions are therefore sometimes difficult to separate. The interaction between Cos2 and Fu has several roles that depend on the Hh signaling environment (Chen and Jiang, 2013). It has a general role in maintaining stability of both proteins (Liu et al., 2007; Lum et al., 2003b). Cos-Fu interaction is also necessary for efficient processing of Ci-155 without Hh (Lefers et al., 2001; Methot and Basler, 2000) and for transducing Smo activation (Shi et al., 2011; Zhang et al., 2011; Zhou and Kalderon, 2011). However, the lack of appropriate reagents has prevented further exploration of its significance for Ci-155 activation (Ruel et al., 2007; Zhou and Kalderon, 2010). We used Fu deletion variants and Cos2 with specific alterations in Fu binding or amino acids that are phosphorylated by Fu at different levels of expression to confirm what is known about the role of Cos2 in Ci-155 processing and to answer what is the role of Cos2 in Ci-155 activation (with an emphasis on Cos2/Fu association).

Effect of Cos2-Fu and Cos2-CORD interactions on Ci-155 processing

We found that binding between Fu and Cos2 is required for efficient processing of Ci-155 by using *fu* alleles with truncations that prevent binding to Cos2 (Robbins et al.,

1997) and by expressing a variant of Cos2 with impaired binding to Fu at natural levels. Truncated Fu proteins from the alleles fu^{M1}, fu^{RX2}, and fu^{W3}, resulted in a cell-autonomous increase in the levels of Ci-155 in anterior *fu* clones, consistent with a severe defect in Ci-155 processing (Figs. 3.1J, 3.S1A, B), although some processing continued in posterior *smo* fu^{M1} clones sufficient to repress *hh-lacZ* reporter (Fig. 3.1L). Repression of *hh-lacZ* reporter is a more sensitive assay for detecting residual Ci processing in P cells that express *ci* cDNA transgene (Methot and Basler, 1999; Smelkinson et al., 2007). Similarly, expressing a Cos2ΔFu transgene under the control of genomic *cos2* regulatory sequences did not prevent the accumulation of Ci-155 in anterior *cos2* clones (Fig. 3.4E), although the transgene supported *hh-lacZ* repression in posterior *smo cos2* clones ectopically expressing Ci (Fig. 3.S4A). These findings indicate that Cos2 binding to Fu is necessary for efficient Ci-155 processing.

Defects in Ci-155 processing by Cos2ΔFu could be offset by expressing very high levels of Cos2ΔFu (Fig. 3.2D-F), but excess Cos2 did not reduce Ci-155 accumulation in fu^{M1} clones (Fig. 3.1K). The most likely explanation is that residual binding between Cos2ΔFu and Fu allows sufficient Fu to be recruited to Ci-155 when Cos2ΔFu is expressed in excess but that Fu must be recruited to the Ci-155 complex for Ci-155 to be processed efficiently.

Su(fu) is a potential participant in Ci-155 processing because it can bind to both Ci-155 and Fu (Monnier et al., 1998; Methot and Basler, 2000), and therefore might aid Fu recruitment. Although Su(fu) has no apparent effect on Ci-155 processing in Drosophila when Cos2 and Fu are present (Ohlmeyer and Kalderon, 1998), western blotting and antibody analysis of mouse embryonic tissue demonstrated that removal of mouse Sufu

reduces the level of Gli repressors resulting in de-repression of Shh transcription targets (Svärd et al., 2006; Wang et al., 2010). We therefore tested whether Su(fu) might contribute in Drosophila when Ci-155 processing was compromised by alterations to Fu or Cos2. We found that removing Su(fu) did not prevent *hh-lacZ* repression (and hence residual Ci-155 processing) in fu^{M1} *smo* clones or in *smo cos2* clones co-expressing excess Cos2ΔFu with either Ci or CiΔΔ (Fig. 3.3). Furthermore, Ci-155 levels were not increased in *cos2* mutant clones expressing excess Cos2ΔFu even when Su(fu) was absent (data not shown). Hence, we found no role for Su(fu) in promoting Ci-155 processing.

What is the significance of Cos2 binding to Fu for Ci-155 processing? We found that S2 cells lysates contained less HA-Fu if co-transfected with Flag-Cos2ΔFu rather than wild type Flag-Cos2 (Fig. 3.2A). Levels of Fu were also markedly reduced in *cos2* clones (Lum et al., 2003b) as in *cos2* clones expressing Cos2ΔFu (Fig. 3.2C). Thus, the interaction with Cos2 ensures stability of Fu. Fu may also stabilize Cos2 based on reduction of Cos2 levels in Kc cells treated with Fu RNAi (Liu et al., 2007), but analysis with Cos2 antibody showed significant accumulation of UAS-Cos2ΔFu transgenes in *cos2* clones (Fig. S2I, J). Also, because Cos2 is known to bind to both Fu (Robbins et al., 1997) and Ci (Wang, G. et al., 2000) and because the other potential adaptor between Fu and Ci, Su(fu) (Monnier et al., 1998), does not have an apparent role in Ci-155 processing, binding to Cos2 is likely to be required for recruitment of Fu to Ci-155. The simplest explanation for this requirement is that Fu makes an essential contribution to the scaffolding function of Cos2 (Zhang et al., 2005) by binding to one or more protein kinases, PKA, CK1 and GSK3 (Fig. 4.1).

We studied the importance of Cos2 binding to the CORD region of Ci using Cos2-S182N, a variant with a detrimental mutation in a residue thought to be necessary for ATP hydrolysis in kinesins (Ho et al., 2005). Cos2 is an atypical kinesin that exhibits some evidence of microtubule-based motility in tissue culture cells, which requires ATP hydrolysis and is prevented by the S182N mutation (Ho et al., 2005; Farzan et al., 2008). These movements may be relevant in Hh-free regions because they require Cos2 association with MTs, which is opposed by Hh (Robbins et al., 1997). The purpose of this motility function is not well-understood. The S182N substitution was also previously shown to affect binding of Cos2 to the CORD region of Ci, suggesting a second possible function for nucleotide binding (Zhou and Kalderon, 2010). ATP potentiates the in vitro interaction between Cos2 and CORD, which is lost for Cos2-S182N (Zhou and Kalderon, 2010). Surprisingly, the absence of the CORD region facilitated the reduction of Myc-Ci levels by excess Cos2-S182N in *smo cos2* clones, suggesting that CORD normally inhibits Ci-155 processing and that binding of CORD by Cos2 relieves this inhibition (Zhou and Kalderon, 2010). We used Cos2-S182N under the control of genomic sequences (gCos2-S182N) to confirm that binding of Cos2 to CORD promotes Ci-155 processing. Indeed, gCos2-S182N was defective in processing of Ci-155, producing Ci-155 accumulation in anterior wing discs that were often enlarged (Fig. 3.4B) possibly because of de-repression of the *dpp* transcription target (Methot and Basler, 2001), and did not prevent cell-autonomous Ci-155 elevation in *cos2* clones (Fig. 3.4E). As with excess Cos2-ΔFu, gCos2-S182N was capable of some processing sufficient for *hh-lacZ* repression in *smo cos2* clones (Fig. 3.S4B). Also, gCos2-S182N did not prevent the induction of ectopic *ptc-lacZ* in *cos2* clones, reducing the level of *ptc-lacZ* expression only slightly compared to *cos2*

clones without the transgene (Fig. 3.4I), which suggests that Cos2-S182N is defective in silencing excess Ci-155. Therefore, these studies are consistent with the previous hypothesis that the interaction between Cos2 and CORD is important for Ci-155 processing, and also suggest that Cos2-CORD interaction is important for limiting the activity of Ci-155. In order to prove that the defects of gCos2-S182N are due to a failure to bind Ci-155 it will be important to test the properties of gCos2-S182N in *cos2* mutant clones that also exchange endogenous Ci for CiΔΔ expressed at normal levels.

The Role of Cos2 in Responding to Hh; Fu Activation Requires Interaction with Cos2

We next asked if binding of Cos2 to Fu is important for responses to Hh. It was previously shown that each of these proteins is required for induction of anterior En at the AP border region of active Hh signaling, which was blocked in *cos2* clones and in fu^{mH63} clones with inactive Fu kinase (Wang, G. et al., 2000; Ohlmeyer and Kalderon, 1998). Further analysis of the interaction was complicated by higher than endogenous expression levels of *UAS-Cos2* transgenes, which inhibit Hh signaling at the AP border (Zhou and Kalderon, 2010). We found that a wild type Cos2 transgene under control of genomic regulatory sequences (gCos2) supported a normal response to Hh, rescuing the expression of En and *ptc-lacZ* reporter in *cos2* clones at the AP border (Fig. 3.4G). Expressing gCos2ΔFu instead of wild type gCos2 in *cos2* clones at the AP border produced defects in activation that resembled the ones previously observed in fu^{mH63} clones (Ohlmeyer and Kalderon, 1998; Zhou and Kalderon, 2011), which included loss of anterior En expression, reduction in *ptc-lacZ* expression (Fig. 3.4H) and accumulation of Ci-155 (Fig. 3.4E), possibly due to a block in Cul3-dependent Ci-155 degradation that occurs in high Hh

regions (Zhang et al., 2006). These findings provided the most direct evidence to date for the importance of Cos2 binding to Fu in allowing the activation of Fu kinase.

Hh activates Fu by activating Smo, and expressing activated Smo (SmoD123) can induce strong ectopic En expression in anterior wing discs (Zhou and Kalderon, 2011). Mutational analysis, co-localization and FRET experiments in wing discs and tissues culture cells showed that the Hh activity gradient translates into a gradient of Smo activation with progressive changes in Smo phosphorylation (Apionishev et al., 2005; Zhou et al., 2006), conformation or clustering (Zhao et al., 2007; Shi et al., 2011; Fan et al., 2012) and membrane accumulation (Denef et al., 2000; Jia et al., 2003; Zhao et al., 2007). We explored whether Cos2-Fu interaction was necessary specifically for Fu activation, for Smo activation or for both.

First we found that gCos2ΔFu failed to induce *ptc-lacZ* expression in *smo cos2* clones in response to SmoD123, suggesting that gCos2ΔFu is defective in communicating the activation status of Smo (Fig. 3.5B). Deletion analyses in S2 cells showed that Cos2 can interact directly with the Smo C-terminal tail, anchoring Fu at the membrane (Jia et al., 2003). Synthetically anchoring Fu at the membrane through a palmitoylated GAP-43 domain produces a constitutively active GAP-Fu variant that results in strong Hh pathway activation independent of Fu association with Smo (Claret et al., 2007; Zhou and Kalderon, 2011). Expressing high levels of GAP-Fu induced *ptc-lacZ* expression in *smo cos2* clones with gCos2ΔFu (Fig. 3.5E), suggesting that the role of interaction with Cos2 for Fu activation can be compensated by concentrating Fu at the membrane. FRET analyses in S2 cells confirmed that proximity between the N-termini of Fu molecules tagged with either CFP or YFP was promoted by co-transfection with Flag-Cos2 and decreased by treatment

with siRNA against Cos2 (Shi et al., 2011). Clustering was suggested to stimulate cross-phosphorylation at Ser159 between different molecules of Fu (Fukumoto et al., 2001; Zhou and Kalderon, 2011; Shi et al., 2011). Preventing cross-phoshorylation by using Fu-Ser159A or disrupting Fu interaction with Cos2 by using Fu 1-473 which lacks the Cos2-binding domain (Robbins et al., 1997) precludes the rescue of *ptc-lacZ* and En at the AP border of fu^{mh63} wing discs (Zhou and Kalderon, 2011). Thus, cross-phosphorylation between Fu molecules was previously argued to be necessary for Fu kinase activation and to be stimulated by association of inactive Fu molecules with Cos2 (Shi et al., 2011; Zhang et al., 2011; Zhou and Kalderon, 2011). Our evidence that Cos2ΔFu fails to allow Fu activation by activated Smo provides further evidence that cross-phosphorylation between Fu molecules requires that they bind to Cos2 (Fig. 4.1).

We also tested the effect of binding between Cos2 and Fu on Smo accumulation by immunostaining *cos2* wing discs that express gCos2ΔFu with Smo antibody. The normal pattern of Smo expression with increased protein stability in high Hh signaling regions (posterior and AP border) was supported by Cos2ΔFu substitution, suggesting that Cos2-Fu interaction is not required for the accumulation component of Smo activation (Denef et al., 2000; Jia et al., 2003; Zhao et al., 2007).

Cos2 Variants with no Apparent Defect in Response to Hh

We found several variants of gCos2 with no clear defect in responding to Hh. Genomic Cos2-S182N (gCos2-S182N) supported expression of *ptc-lacZ* and anterior En at the AP border of *cos2* discs and in *cos2* clones (Fig. 3.4I). Also, gCos2-S182N did not affect Smo accumulation in *cos2* discs (Fig. 3.S5C) suggesting that the S182N defect in

ATP hydrolysis does not affect the stabilization component of Smo activation (Denef et al., 2000; Ho et al., 2005). Thus, Cos2-S182N has a defect in stimulating Ci-155 processing and in limiting the activity of Ci-155 in the absence of Hh but no clear defect in responding to Hh.

Prior studies suggested that Fu-dependent phosphorylation of Cos2 is important for a normal response to Hh and for the ability of Fu kinase activity to oppose processing of Ci-155. A Ci transcriptional activity assay with a *ptc*-luciferase reporter in Cl8 cells demonstrated that Cos2 S931A prevented a normal positive response to Fu kinase, suggesting that Fu-dependent phosphorylation of S931 is important for activation of Ci-155 (Ranieri et al., 2012). The cells were transfected with 10 ng of Cos2, Fu and Smo transgenes, each under control of the strong Actin-5C promoter, producing high non-physiological levels of the three proteins. Earlier, the same group established that Cos2 S572D did not reduce the level of Ci-155 in *cos2* clones as efficiently as wild type Cos2, implicating Fu-dependent phosphorylation of S572 in the inhibition of Ci-155 processing (Ruel et al., 2007). The UAS-Cos2 transgenes were expressed from cDNA constructs under control of 71B Gal4 promoter that drives expression in the wing pouch, resulting in non-physiological and non-uniform levels of transgenic proteins. These findings differed from the observations of genomic Cos2 transgenes (Figs. 3.6, 3.S6). When expressed at physiological levels, Cos2 S931A, S572A, S572AS931A, and S572D supported the expression of *ptc-lacZ* and anterior En in *cos2* clones at the AP border, indicating a normal positive response to Hh (Fig. 3.6A-D). Moreover, these genomic transgenes fully reduced the level of Ci-155 in *cos2* clones, consistent with normal processing of Ci-155 (Fig. 3.S6A-D). Finally, each transgene rescued *cos2* homozygous animals to adulthood,

suggesting that, contrary to prior observations, Fu-dependent phosphorylation of Cos2 is not essential for maintaining a normal response to Hh.

In summary, our findings are consistent with the model where interaction between Cos2 and Fu helps scaffold PKA and other kinases and communicates the activation status of Smo to Fu for Ci-155 activation in Drosophila (Fig. 4.1). Binding to Cos2 has an additional role in maintaining adequate levels of Fu, perhaps to ensure the stability of this interaction (Lum et al., 2003b; Ruel et al., 2003). Kif7 mammalian homolog of Cos2 is important for processing and activation of Gli proteins in mice (Cheung et al., 2009; Hsu et al., 2011). Without Kif7, mouse embryos show phenotypes consistent with pathway activation and thwarted development of embryonic floor plate (Cheung et al., 2009). Kif7 is not known to associate with an ortholog of Drosophila Fu (Endoh-Yamagami et al., 2009) which could recruit processing-promoting kinases (e.g., PKA) to Ci-155 in Drosophila. Moreover, targeted disruption of Fu ortholog is not known to cause any defects in Hh signaling in mammals (Chen et al., 2005; Merchant et al., 2005; Wilson et al., 2009). However, PKA requirement is conserved for processing of Gli3, the main source of Hh transcription repressor in mammals (Wang, B. et al., 2000). Western blot analysis demonstrated that mutating key PKA target sites on Gli3 prevented generation of the Gli3 repressor in tissue culture cells (Wang, B. et al., 2000). Therefore, Kif7 likely recruits PKA to Gli3 through a different route.

Ectopic activation of Hh target genes in Sufu knockouts (embryonic lethal) suggested that Sufu is an essential negative regulator of Hh response in mammals (Svärd et al., 2006). Binding analyses in mouse embryonic cells showed that Sufu binds Gli3 (Ding et al., 1999) and Gsk3β (Kise et al., 2009), and Sufu may also interact with PKA (Svärd et

al., 2006). IP and western blot analyses of extracts from Shh-treated mouse embryonic cells demonstrated that Shh promotes dissociation of full-length Gli3 from Sufu/Gsk3β complexes, blocking the generation of Gli3 repressor (Kise et al., 2009). Thus, the role of Sufu in mammalian Hh pathway can be partly explained through its ability to bind processing-promoting kinases and is likely performed by Fu in Drosophila (Svärd et al., 2006; Kise et al., 2009).

Our data with genomic Cos2 transgenes are consistent with the prior suggestion that Fu must phosphorylate S572 in order to inhibit Ci-155 processing (Zhou and Kalderon, 2011). We found that gCos2-S572A (alone or together with S931A) did not support any elevation of Ci-155 levels in response to activated Fu, whereas wild-type gCos2 and gCos2-S931A did allow a slight increase in Ci-155 levels (Fig. 3.6I-L). Although Fu can stabilize Ci-155 Fu kinase activity is not required for Hh to inhibit Ci-155 processing at the AP border (Ohlmeyer and Kalderon, 1998; Zhou and Kalderon, 2011).

Cos2 phosphorylation by Fu may also have a role in promoting stability of Fu, perhaps by reinforcing the interaction between Cos2 and Fu. Immune precipitation analysis of Smo-Cos2-Fu complex followed by western blotting with phospho-specific antibody against Ser572-P demonstrated that extracts of Hh-treated Kc cells positive for Cos2-Ser572P precipitate different amounts of Fu protein with IP for Cos2 compared to Fu IP, suggesting that phosphorylation destabilizes the Cos2-Fu interaction (Ruel et al., 2007). Also, the HA-Cos2-S572A transgene products that cannot be phosphorylated on Ser572 fail to accumulate to the level of wild type HA-Cos2 in Hh-treated S2 cells (Liu et al., 2007). Therefore, Cos2 phosphorylation may promote stability of Cos2-Fu interaction, and thereby the stability of Fu. Limiting the concentration of Fu protein through requirement

for Cos2 binding may prevent unregulated pathway activation due to clustering of Fu molecules (Zhou and Kalderon, 2011).

Thus, although phosphopeptide antibodies to two Fu target sites on Cos2 were used to demonstrate graded activity of Fu kinase at the AP border (Ranieri et al., 2012), these Fu-dependent phosphorylation sites are not essential for a normal response to Hh and cannot be the sole targets of Fu kinase. Su(fu) protein is also known to contain Fu-dependent phosphorylation sites and was thought to be a second major target of Fu kinase in addition to Cos2 (Zhou and Kalderon, 2011). However, mutating phosphorylation sites on both Su(fu) and Cos2 did not disrupt the Hh responses in *cos2* clones at the AP border where the protein variants were used to substitute for endogenous proteins (data not shown). Therefore, it is likely that Fu kinase mediates Hh response redundantly through other yet unidentified targets.

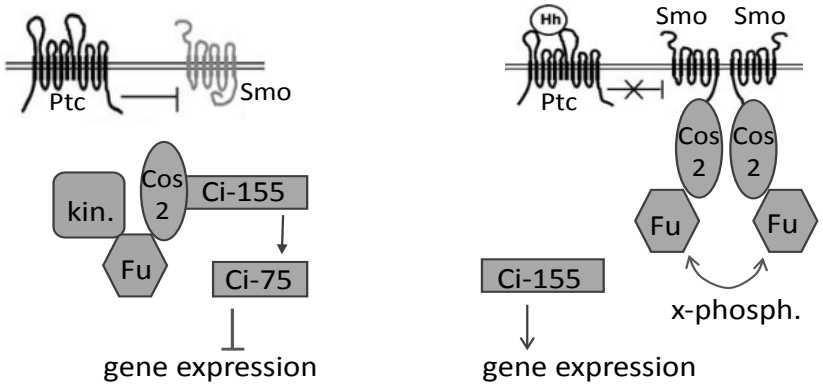


Figure 4.1. Summary of Findings and Possible Roles of Cos2-Fu Interaction in Hh Signaling (adapted from Zhang et al., 2005).

Without Hh: We found that Cos2-Fu interaction is important for efficient processing of Ci-155, possibly because Fu scaffolds processing-promoting kinases (kin.) as was previously suggested for Cos2 (Zhang et al., 2005). We also confirmed that Cos2-Fu interaction is important for stability of Fu (Lum et al., 2003b). We

used gCos2-S182N to confirm that ATP-dependent interaction between Cos2 and CORD region of Ci-155 relieves inhibition of Ci-155 processing by CORD (Zhou and Kalderon, 2010). At moderate levels of Hh (not shown): We found that Cos2-CORD interaction limits the activity of Ci-155, possibly by retaining Ci-155 in the cytoplasm (Wang et al., 2000). We confirmed that Fu-dependent phosphorylation of Cos2 stabilized Ci-155 (Zhou and Kalderon, 2011). At high levels of Hh: We found that Cos2-Fu interaction is essential for Fu activation, possibly because the interaction between Cos2 and inactive Fu molecules stimulates Fu cross-phosphorylation (x-phosph., double arrow) (Shi et al., 2011; Zhang et al., 2011; Zhou and Kalderon, 2011). Consistent with this hypothesis, gCos2ΔFu did not respond to synthetically active Smo (SmoD123; (Jia et al., 2004)).

Concluding Remarks

We developed a set of Cos2 transgenes that allowed us to study the behavior of Cos2 variants expressed at physiological levels in place of the normal Cos2 gene, either in clones of marked cells or throughout all cells of a fly. Prior studies of Cos2 had not used this approach. In fact, earlier structure-function studies of Cos2 relied either on the expression of UAS-Cos2 cDNAs, which were invariably expressed at excessively high levels in fly tissues, or on over-expressed transgenes assayed in tissue culture cells, often together with other over-expressed proteins. Those earlier studies provided plenty of evidence that Cos2 expression levels are in fact very important for the normal function of Cos2 in Hh signaling (Zhou and Kalderon, 2010; Raisin et al., 2010; Ranieri et al., 2012). Most strikingly, both in Drosophila wing discs and in tissue culture cells, Cos2 over-expression strongly diminishes Hh responses (Zhou and Kalderon, 2010; Raisin et al., 2010). Thus, our approach was absolutely essential to be able to measure the role of Cos2 variants in responding to Hh and provided the most definitive measures to date of

physiologically-relevant Cos2 function in the absence of Hh signaling. The properties of a Cos2 variant with strongly impaired Fu binding (Cos2ΔFu) provided a clear example of the importance of Cos2 expression levels. When expressed at physiological levels Cos2ΔFu supported only minimal rates of Ci-155 processing (Fig. 3.4B, E), whereas over-expression supported normal, efficient Ci-155 processing (Fig. 3.2E, F).

Using this approach of expressing Cos2 variants at physiological levels, coupled to re-examination of the properties of a set of *fu* alleles, we were able to conclude that Cos2 must recruit Fu to processing complexes in order for Ci-155 to be processed efficiently to Ci-75 in the absence of Hh. Moreover, even when Cos2-Fu association was compromised Su(fu) appeared to play no role in supporting Ci-155 processing, despite its theoretical potential to bring Fu to Ci-155 (Fig. 3.1M). These data substantiate earlier evidence that Fu has a role in Ci-155 processing (Methot and Basler, 2000; Lefers et al., 2001), which we found cannot be compensated by excess Cos2, whereas Su(fu) does not. In mammals, the Fu ortholog plays no role in Hh signaling while the Su(fu) ortholog is thought to support the Cos2 ortholog Kif7 in processing Gli proteins (Svard et al., 2006). The simplest explanation for these findings is that Fu in Drosophila, and SuFu in mammals binds to one or more of the protein kinases, PKA, CK1 and GSK3 that Cos2/Kif7 proteins help to scaffold to Ci/Gli proteins. Indeed, SuFu has been shown to be capable of binding to GSK3 (Kise et al., 2009).

We also gained some insight into Cos2-Ci associations. It was previously shown that three regions of Ci (CDN, Zinc fingers and CORD) could interact with Cos2 in vitro (Wang and Jiang, 2004; Zhou and Kalderon, 2010). Only removal of both the Zinc finger

and CORD regions of Ci-155 blocked Ci-155 processing, suggesting that either of these regions could suffice for recruiting Cos2-Fu and associated kinases (Zhou and Kalderon, 2011). It was nevertheless speculated that Cos2 binding to the CORD domain also had a unique function, based on the properties of a Cos2 variant (S182N) deficient for CORD domain (but not Ci zinc finger region) binding (Zhou and Kalderon, 2010). We confirmed that Cos2 S182N expressed at physiological levels supported only inefficient Ci-155 processing (Fig. 3.4C, F), supporting the earlier hypothesis. Moreover, we found that Ci-155 spared from processing was partially active (in contrast to the higher levels of Ci-155 spared by Cos2ΔFu), suggesting that Cos2-CORD interaction not only promotes Ci-155 processing but also limits Ci-155 activity, perhaps by limiting nuclear access (Fig. 3.4H, I).

By testing responses to Hh supported by Cos2 variants we found that Cos2 must bind to Fu in order for Ci-155 to be fully activated in response to either Hh or activated Fu. This finding provides further evidence that Cos2 binding to Fu allows Fu molecules to cross-phosphorylate and establish an active protein kinase conformation in response to activated Smo (Shi et al., 2011; Zhang et al., 2011; Zhou and Kalderon, 2011).

A second finding, however, contradicts important earlier publications concerning the role of Cos2 in transmitting Hh signals (Raisin et al., 2010; Ranieri et al., 2012). These earlier studies used Ci activity assays in tissue culture to measure the consequences of altering two Fu phosphorylation sites on Cos2. To compensate for the inhibitory effects of excessive concentrations of Cos2 used in these assays, the authors simultaneously increased Smo protein to carefully titrated optimal levels and then tested responses to over-expressed Fu kinase. The conclusion was that both S572 and S931 phosphorylation are

important for transduction of Hh signal. By contrast, we found that a Cos2 transgene lacking both residues and expressed at physiological levels in a *cos2* null mutant background fully rescued all responses to Hh and Fu, supporting development of whole, morphologically normal, fertile flies. While our results are clearly the most relevant and definitive, at least two major questions arise. How is it that Cos2 is phosphorylated at S572 and S931 in vivo, as has clearly been demonstrated, if it serves no purpose at all? Also, shouldn't there be some in vivo correlate of the results obtained in tissue culture? One possible resolution could be if the effects of Cos2 phosphorylation played only a very small role that was not evident by examining in vivo patterning but is detectable by quantitative assays in tissue culture. Related to this, perhaps the consequences of Cos2 phosphorylation would be more evident if other responses were compromised (and they may indeed be compromised in the tissue culture assays). We tested this idea in one way. Another possible target of Fu, Su(fu), affects the response to Hh in the absence of Fu kinase and is thought to limit the activity of Ci possibly by acting as a cytoplasmic anchor (Ohlmeyer and Kalderon, 1998; Wang, G. et al., 2000). Cos2 is also known to anchor Ci in the cytoplasm through a CORD binding domain from the results of LMB experiments in Cl8 cells (Wang, G. et al., 2000), and the specific defect in CORD binding exhibited by Cos2-S182N mutant is likely to have caused the ectopic *ptc*-lacZ induction in anterior *cos2* clones with gCos2-S182N (Fig. 3.4I). Therefore, Fu could be targeting Cos2 redundantly with Su(fu) to antagonize cytoplasmic tethering of Ci by these proteins. Fu phosphorylation sites on Su(fu) have been mapped but alteration of those residues was found to have no effect on Hh signaling. When we altered all known Fu targets sites on Su(fu) together with those on Cos2 we still observed a normal response to Hh in *cos2*

clones at the AP border (data not shown). Thus, if a function of Cos2 phosphorylation (or Su(fu) phosphorylation) is disguised by another response to activated Fu we must first identify that target and then test for potential redundancy. Ci is one potential target but no Fu sites have yet been mapped on Ci.

Chapter V

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